The neuroprotective role of osteopontin in in-vitro and in-vivo models of Parkinson's disease

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The neuroprotective role of osteopontin in *in-vitro* and *in-vivo* models of Parkinson's disease

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Certificate

This is to certify that research work embodied in this thesis entitled "The neuroprotective role of osteopontin in in-vitro and in-vivo models of Parkinson's disease" has been carried out by me under supervision and guidance of Prof. Peter Jenner and Dr. Sarah Salvage.

Sara Ailane

Abstract

Osteopontin (OPN), a glycosylated phosphoprotein, is down regulated in remaining dopaminergic neurones in Parkinson's disease. It has protective properties including anti-inflammatory and anti-apoptotic effects. Therefore, it was hypothesised that OPN treatment protects dopaminergic neurons from toxin induced cell death, and its endogenous expression in cells confers intrinsic protection. Consequently, OPN was investigated for neuroprotective effects in toxin models of dopaminergic cell death using SH-SY5Y and N1E-115 cell lines with different OPN expression phenotypes, primary E14 ventral mesencephalic (VM) culture and rats.

Cell lines expressed receptors linked to the pro-survival effects of OPN ($Ig\alpha_v$, $Ig\beta_3$, $Ig\beta_1$ and CD44). However, endogenous OPN expression did not affect vulnerability of cell lines to the toxins MPP^+ or H_2O_2 and exogenous pre-treatment with OPN did not protect them from toxin-induced cell death. By contrast, pre-treatment of VM cultures with OPN protected them against MPP^+-induced dopaminergic cell loss. Importantly, a 15-mer peptide fragment of OPN containing the RGD integrin binding site retained protective properties of OPN. Further, treatment with the integrin receptor antagonists, RGDS and GRGDSPK, prevented OPN's protective effect, suggesting a role for integrins in the protective effect. OPN induced an increase in the number of microglia in VM cultures but the role of glial cells in OPN's protective effect is not fully clear. In the LPS lesioned rats, there was an up-regulation of the expression of $Ig\alpha_v$, $Ig\beta_3$, $Ig\beta_1$ and CD44 receptors and prior intra-nigral OPN injection protected dopaminergic cells of the substantia nigra against LPS toxicity. Immunohistochemical investigation revealed that OPN significantly decreased the inflammatory microgliosis produced by LPS.

In conclusion, OPN was protective against toxin induced cell death in primary VM culture *in vitro* and inflammation mediated cell death *in vivo*, but not in immortalised cell lines. The protective effects of OPN were mediated through an effect on integrin receptors and microglial cells.

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List of abbreviations

3-NT	3-nitrotyrosine
6-OHDA	6-hydroxydopamine
8-HDG	8-hydroxy-2-deoxy-guanosine
AAV	Adino ascociated virus
ABC	Avidin biotin complex
AD	Alzheimer's disease
ADAGIO	Attenuation of disease progression with azilect given once-daily
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BBB	Blood brain barrier
bcl-2	B-cell lymphoma-2
Bcl-X_L	B-cell lymphoma-extra large
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
CALM-PD	Comparison of the dopamine agonist pramipexole versus Levodopa on motor complications of Parkinson's disease
CMA	Chaperon mediated autophagy
CNS	Central nervous system
CO_2	Carbon dioxide
COMT	Catechol-O-methyl transferase
CoQ10	Coenzyme Q10
COX	Cyclooxygenase
CR3	Complement receptor 3
CSF	Cerebrospinal fluid
CV	Cresyl violet
DAB	3.3'-diaminobenzidene
DAPI	4',6-diamidino-2-phenylindole
DAT	Dopamine transporter

DATATOP	Deprenyl and tocopherol antioxidative therapy of parkinsonism
DIV	Day *in-vitro*
DMEM	Dulbecco's modified eagle's medium
DMNV	Dorsal motor nucleus of the vagus
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
dUTP	Deoxynucleotidyl transferase-mediated
E14	Embryonic day 14
EC_{50}	Median effective concentration
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal-regulated kinases
Eta-1	Early T lymphocyte activation-1
FBS	Foetal bovine serum
FK-506	Tacrolimus
GABA	Gamma-aminobutyric acid
GAD	Glutamate decarboxylase
GAGs	Glycosaminoglycans
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDNF	Glial derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GFP	Green fluorescent protein
H_2O_2	Hydrogen peroxide
HIV	Human immunodeficiency virus
Hsc70	Heat-shock cognate 70
IF	Immunofluorescence
IFN-γ	Interferon-γ
Ig	Integrin
IgG	Immunoglobulin G
IgM	Immunoglobulin M

Igα_v	Integrin α_v
Igβ_1	Integrin β_1
Igβ_3	Integrin β_3
IL	Interleukin
ILBD	Incidental lewy body disease
iNOS	Inducible nitric oxide synthase
IP	Immunoperoxidase
JNK	c-Jun N-terminal kinase
KO	Knock out
LB	Lewy body
LC	Locus coeruleus
LDH	Lactate dehydrogenase
L-DOPA	L-3,4-dihydroxyphenylalanine
LPS	Lipopolysaccharide
LRRK2	Leucine-rich repeat kinase 2
MAC-1	Macrophage antigen complex-I
MAO-B	Monoamine oxidase-B
MAPK	Mitogen-activated protein kinases
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MPP^+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial DNA
NaC1	Sodium chloride
NADH	Nicotinamide dehydrogenase
NBM	Nucleus basalis of meynert
NeuN	Neuronal nuclei
NF-$_K$B	Nuclear factor-kappa B
NGF	Nerve growth factor
NINDS	National institute of neurological disorders and stroke
NK	Natural killer
NO	Nitric oxide

NOS	Nitric oxide synthase
NSAID	Non-steroidal anti-inflammatory
OD	Optical density
OPN	Osteopontin
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PD	Parkinson's disease
PFA	Paraformaldehyde
PINK1	PTEN-induced putative kinase 1
PMSF	Phenylmethylsulfonyl fluoride
PPARγ	Peroxisome proliferator-activated receptor-γ
PRECEPT	Parkinson research examination of CEP1348 trial
PROUD	Pramipexole on underlying disease
PSN	Penicillin, streptomycin and neomycin
PVDF	Polyvinylidene fluoride
REAL-PET	Requip as early therapy versus L-dopa-positron emission tomography
RN	Raphe nuclei
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SN	Substantia nigra
SNpc	Substantia nigra pars compacta
SNpr	Substantia nigra pars reticulata
TBS	Tris buffered saline
TBS-T	Tris buffered saline-Triton-X 100
TEMED	N,N,N',N'-Tetramethylethylenediamine
TEMPO	TVP-1012 in early monotherapy for Parkinson's disease outpatients
TH	Tyrosine hydroxylase
Th1	T helper cell
TM	Transmembrane

TNF-α	Tumour necrosis factor-α
TUNEL	Transferase-mediated dUTP nick end-labeling
UCHL-1	C-terminal hydroxylase-L1
UPDRS	Unified Parkinson's disease rating scale
UPS	Ubiquitin-proteasome system
VM	Ventral mesencephalic
VMAT2	Vesicular monoamine transporter 2
VTA	Ventral tegmental area

Publications

Abstracts

Ailane,S., Rose, S., and Jenner P. (2009). Osteopontin protects primary dopaminergic neurons but not cell lines from toxin induced cell death. Poster communication at Society for Neuroscience.

Ailane,S., Rose, S., and Jenner P. (2009). Expression of Integrins α_v, β_3, β_1 and CD44 receptor may explain the neuroprotective effects of Osteopontin. Poster communication at XVII WFN World Congress of Parkinson's Disease and Related Disorders.

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Chapter 1 Introduction

1.1 Parkinson's disease

Parkinson's disease (PD) is a progressive neurodegenerative disease characterised by the cardinal symptoms of bradykinesia, rigidity, resting tremor, gait dysfunction and postural instability (Jankovic, 2008). Non-motor symptoms can also occur including depression, anxiety, autonomic dysfunction, cognitive impairment, and sleep disturbance (Jankovic, 2008). PD is the second most common neurodegenerative disorder after Alzheimer's disease. It affects 1% of the population over 60 years of age and the incidence increases with age (Alves *et al.*, 2008).

Current treatments available for PD focus on alleviating motor symptoms by replacing striatal dopamine and are, therefore, only symptomatic in nature. Levo-DOPA (L-DOPA), the amino acid precursor of dopamine, is the most effective treatment available (Halkias *et al.*, 2007; Rezak, 2007; Singh *et al.*, 2007). It is used in combination with the peripheral dopa-decarboxylase inhibitor, carbidopa to reduce peripheral metabolism allowing a reduction in L-DOPA dose and limiting side effects such as nausea, vomiting and hypotension. Dopamine agonists are used as adjunct to L-DOPA therapy to reduce motor fluctuations but are also widely used as first line therapy in early disease and can delay the need for L-DOPA for up to 5 years (Lees, 2005). Monoamine oxidase-B (MAO-B) inhibitors, selegiline and rasagiline are used early in the treatment to reduce endogenous dopamine metabolism and in later disease to potentiate the effects of L-DOPA (Schapira, 2009b). Inhibitors of catechol-O-methyl transferase (COMT) such as entacapone and tolcapone are also used to prevent peripheral L-DOPA metabolism so maximising delivery to the brain. However, motor complications or fluctuations (dyskinesia, "ON" and "OFF" periods and wearing off effect) develop in 40% of patients after 4-6 years of L-DOPA therapy and 70% of patients after 9 years (Ahlskog *et al.*, 2001) and can cause significant social and functional disability (Pahwa *et al.*, 2006; Rezak, 2007; Singh *et al.*, 2007).

Therefore there is a clear need for a neuroprotective agent that can prevent further loss of dopaminergic neurones from the substantia nigra (SN) and ultimately stop progression of the disease. There are currently no proven treatments for PD that

act to stop or slow progression of the illness although development of neuroprotective strategies is on-going.

1.1.1 Neuroprotective therapies in PD

Many drugs have been tested for their neuroprotective effects including dopamine agonists, MAO-B inhibitors, anti-apoptotic agents and neurotrophic factors (Table 1.1) but so far no drug has been proven to slow progression of the disease (Lohle *et al.*, 2010; Schapira, 2008b). Some examples of drug strategies that have been investigated for neuroprotection are given below.

Despite promising pre-clinical data of neuroprotective properties of MAO-B inhibitors (Olanow *et al.*, 2006; Schapira, 2009a) , clinical trials have not been conclusive as to their benefit. Selegiline was first chosen for investigation based on its capacity to inhibit oxidative stress related to the oxidation of dopamine by MAO-B (Cohen *et al.*, 1989). In a randomised double-blind placebo controlled study (DATATOP), selegiline appeared to slow the progression of motor symptoms of PD (PSG, 1989) but there was insufficient allowance made for its symptomatic effects which biased the initial interpretation of a delay in the time to introduction of L-DOPA (Olanow *et al.*, 1995). Most subsequent studies have shown that after several years of treatments, selegiline treated patients have similar levels of disability to those not receiving the drug (Brannan *et al.*, 1995; Lees, 1995; PSG, 1996). However, there are long term monitoring trials of the effects of selegiline which still appear to show a better outcome than those not receiving the drug (Larsen *et al.*, 1999). Despite controversy over effects of selegiline, another MAO-B inhibitor; rasagiline has also been tested for its ability to slow progression of PD. In a short term clinical trial, where rasagiline was tested in early onset PD patients (TEMPO), motor scores and quality of life measures improved significantly with both dosages used (1 or 2mg daily) compared to placebo-treated control (PSG, 2002a). However, in another study designed to minimise symptomatic effects of rasagiline (1 or 2mg daily) where early treatment with rasagiline was compared to a 6 month-delayed start in a year-long study (ADAGIO) showed different results. Early start treatment with rasagiline 1mg but not 2mg provided a small improvement on the total Unified Parkinson's Disease Rating Scale (UPDRS) (Olanow *et al.*, 2009). The significance

of this benefit is questioned, compared to the annual decline in total UPDRS score in untreated PD (Lohle *et al.*, 2010).

In a similar manner, there is a large body of pre-clinical evidence suggesting that dopamine agonists may be neuroprotective (Schapira, 2002; Schapira, 2009a). Consequently, two main clinical studies have been undertaken in PD patients using the dopamine agonists pramipexole, CALM-PD (Ahlskog, 2005; 2002b) or ropinirole, REAL-PET (Whone *et al.*, 2003). In the first trial, pramipexole treatment achieved a more significant decrease in dopamine transporter loss compared to L-DOPA (Ahlskog, 2005; 2002b). In the second trial, ropinirole treatment resulted in significantly lesser reduction in the uptake of ^{18}F-L-DOPA by SN and putamen compared to L-DOPA (Whone *et al.*, 2003). These results show that dopamine agonists can slow the deterioration of nigro-striatal function as measured by imaging markers, suggesting that these drugs may be protecting against the progressive loss of nigral neurons. However both studies have the criticism of lacking a placebo control group. In addition, these changes in striatal function measured by imaging markers could be due to pharmacological difference between L-DOPA and dopamine agonists in regulating dopamine transporter or ^{18}F-L-DOPA metabolism (Schapira, 2008b). Therefore, at this stage, it cannot be unequivoqually concluded that the observed effects are due to protection against loss of nigral neurons. Consequently, pramipexole entered a new trial (PROUD) using both clinical and imaging end-points in order to clarify the protective effects of the drug (Schapira *et al.*, 2010).

Another type of agents investigated for neuroprotection in PD is anti-oxidants, based on implication of oxidative stress in PD pathogenesis (Section 1.1.4.b). Coenzyme Q10 (CoQ10), a co-factor in the mitochondrial electron transfer chain and a potent anti-oxidant (Echtay *et al.*, 2000; Ernster *et al.*, 1995), is an example of anti-oxidants examined as a potential protective treatment in PD. CoQ10 was investigated for neuroprotection based on its protective effects in MPTP-treated mice (Beal *et al.*, 1998) and in transgenic mice models of Huntington's disease (Ferrante *et al.*, 2002). However, in a randomized, placebo-controlled, double-blind clinical trial in early untreated PD patients using different doses of CoQ10, there was an inverse correlation between dose and degree of clinical change as measured by UPDRS and significant benefit in each treatment group compared to placebo suggesting possible neuroprotection. However, there was no effect on time until L-

DOPA therapy initiation was required or on timed tapping test of motor function (Shults *et al.*, 2002). This trial received the criticism that CoQ10 may be having a symptomatic effect by restoring the impaired energy metabolism of remaining dysfunctional neurons (Hauser *et al.*, 2006). Therefore, a larger multicenter study using CoQ10 was undertaken (Schapira, 2008b) but this was recently stopped due to lack of evidence of any positive or negative effects of CoQ10 (NINDS clinical trials, 2011). Although there is good rationale for using antioxidants as neuroprotective agents, clinical trials have not shown unequivocal data to prove this.

Apoptosis is also implicated in the pathogenesis of PD (Section 1.1.4.a) and this has led to investigation of anti-apoptotic agents as possible neuroprotective therapies for the disease. For example, CEP-1347 is an anti-apoptotic compound that inhibits mixed lineage kinases which induce apoptosis via activation of the JNK pathway (Maroney *et al.*, 1998). *In-vitro* and *in-vivo* pre-clinical data suggested that CEP-1347 may have neuroprotective activity (Silva *et al.*, 2005). Therefore, a clinical trial (PRECEPT) was conducted in early diagnosed patients to test the clinical potential of this agent (PSG, 2007). However, the outcome of the trial was that patients receiving the drug had higher deterioration and required dopaminergic therapy quicker than placebo treated controls (PSG, 2007). This suggested that use of this drug is detrimental rather than protective. Therefore further investigation is required in order to determine the exact role of anti-apoptotic agents as a neuroprotective strategy in PD.

A different approach to altering progression of PD is to not simply stop degeneration of neurons but also to induce a neurorestorative effect. In this respect, a range of neurotrophic factors have been investigated. Glial derived neurotrophic factor (GDNF) has been the most studied neurotrophic factor in PD research as a potential treatment. GDNF protected dopaminergic neurons in 6-OHDA or MPTP lesioned rodents (Hoffer *et al.*, 1994; Lapchak *et al.*, 1997; Tomac *et al.*, 1995), and in MPTP-treated monkeys, resulted in an increase in nigral dopaminergic neurons and an overall improvement in disability as measured by modified UPDRS (Grondin *et al*., 2002). Consequently, a small open label trial where 5 PD patients receiving continuous intraputaminal infusion of GDNF showed significant improvement in symptoms (48% reduction in UPDRS) after one year, and an increase in dopamine storage in the putamen after 18 months (Gill *et al.*, 2003). After the promising

results of the previous trial, a larger, randomised, double blind, placebo-controlled study was carried out to assess the clinical effectiveness of GDNF as a neuroprotective agent (Lang *et al.*, 2006). Results showed that despite a 26% increase in putamen dopamine storage, continuous intraputaminal infusion of GDNF did not result in any significant clinical improvement of motor function. There is considerable evidence for the potential benefits of neurotrophic factors as a treatment of PD. However, the discrepancy in clinical results suggests that more clinical trials are required to clarify the role of GDNF as a potential neuroprotective treatment in PD.

In summary, there have been positive results from pre-clinical studies for some potential neuroprotective agents but in clinical trials, none have shown solid evidence for slowing or stopping progression of the disease by means of protecting against neuronal loss (Lohle *et al.*, 2010; Schapira, 2008b). Thus despite the immense efforts made to find a neuroprotective approach to PD, translation from the laboratory to man has so far failed and new approaches are required. A better understanding of aetiology and pathogenesis of the disease and the mechanisms involved in neurodegeneration may help highlight the properties required for a successful neuroprotectant and this is explored in the forthcoming sections.

Class	Trial	*N*[a]	Primary outcome	Duration
Antiapoptotic agents				
–TCH346	Olanow et al.	301	Time to symptomatic treatment	12–18 months
–CEP-1347	PRECEPT	806	Time to symptomatic treatment	Terminated after ~21 months
–Minocycline	NINDS NET-PD FS-1	200	Change in total UPDRS	12 months
Antioxidants				
–Vitamin E	DATATOP	800	Time to symptomatic treatment	Terminated after ~ 12 months
–Coenzyme Q10	QE2	80	Change in total UPDRS	16 months
	NINDS NET-PD FS-Too	213	Change in total UPDRS	12 months
–Creatine		60	^{123}I-β-CIT SPECT changes	24 months
	NINDS NET-PD FS-1	200	Change in total UPDRS	12 months
Dopamine agonists				
–Pramipexole	CALM-PD	82	^{123}I-β-CIT SPECT changes	46 months
–Ropinirole	REAL-PET	186	^{18}F-DOPA PET changes	24 months
–α-dihydroergocryptine	Pöpperl et al.	25	^{123}I-IPT SPECT changes	52 weeks
Glutamate antagonists				
–Riluzole	Jankovic and Hunter	20	Change in UPDRS II and III	6 months
	Rascol et al.	1084	Time to symptomatic treatment	Prematurely terminated
Levodopa				
–Levodopa	ELLDOPA	361	Change in total UPDRS	40 weeks
MAO inhibitors				
–Selegiline	DATATOP	800	Time to symptomatic treatment	Terminated after ~12 months
	Tetrud and Langston	54	Time to symptomatic treatment	3 years
	SINDEPAR	101	Change in total UPDRS	14 months
	Swedish Parkinson Study Group	157	Time to symptomatic treatment	1–3 years
	Norwegian–Danish Study Group	79	Change in total UPDRS	60 months
–Lazabemide	ROADS	321	Time to symptomatic treatment	12 months
–Rasagiline	TEMPO	404	Change in total UPDRS	12 months
Neuroimmunophilin ligands				
–GPI-1485	NIL-A phase II clinical trial	300	Change in UPDRS motor score	6 months
	NINDS NET-PD FS-Too	213	Change in total UPDRS	12 months
Neurotrophic factors				
–GDNF	ICV GDNF Study Group	50	Change in UPDRS motor score	8 months
	Lang et al.	34	Change in UPDRS motor score	6 months

Table 1.1 Summary of neurorprotection clinical trials in PD (Lohle *et al.*, 2010).

1.1.2 Aetiology of PD

Despite many years of research, the cause of PD remains elusive. Studies have shown that genetic and environmental factors play a role in precipitating PD. The discovery of PD in families (Gasser *et al.*, 1998; Kitada *et al.*, 1998; Kruger *et al.*, 1998; Polymeropoulos *et al.*, 1997) and observation of familial aggregation of PD cases (Cordato *et al.*, 2004; Sveinbjornsdottir *et al.*, 2000) supports the theory of genetic factors playing a role in the aetiology of PD. In fact, mutations in 15 genes (Table 1.2) have been associated with Parkinsonism including:

- SNCA (Kruger *et al.*, 1998; Polymeropoulos *et al.*, 1997) coding a protein (α-synuclein) which can form fibril like aggregates that are found in Lewy bodies (LBs) in familial and sporadic PD (Spillantini *et al.*, 1997). Mutations are shown to increase protein expression (Farrer *et al.*, 2004) which is associated with enhanced protofibril formation (Miller *et al.*, 2004).
- Parkin (Kitada *et al.*, 1998), and ubiquitin C-terminal hydroxylase-L1 (UCHL-1) (Leroy *et al.*, 1998) are both involved with the ubiquitin-proteasome system which degrades unwanted proteins. Studies with knockout (KO) mice suggest that mutations in Parkin may cause mitochondrial dysfunction and oxidative damage (Palacino *et al.*, 2004). UCHL-1 deficient mice showed disturbed re-use of ubiquitin and a resultant accumulation of abnormal proteins in the brain (Saigoh *et al.*, 1999).
- LRRK2 (Paisán-RuIz *et al.*, 2004; Zimprich *et al.*, 2004) a tyrosine kinase-like protein, but the exact function is unknown. Some LRRK2 mutations are associated with an increase in kinase activity (West *et al.*, 2005) and others with decreased GTPase activity (Lewis *et al.*, 2007) but it is not clear how this is involved in PD pathogenesis.
- PINK1 (Valente *et al.*, 2004; Valente *et al.*, 2001), a mitochondrial protein whose function is not known but is shown to be important for neuroprotection against mitochondrial dysfunction and proteasome-induced apoptosis. PPINK1 mutations impair this protective effect (Valente *et al.*, 2004; Valente *et al.*, 2001).
- DJ1 (Bonifati *et al.*, 2003), is suggested to act as an anti-oxidant (Nagakubo *et al.*, 1997) and mutation is associated with increased oxidative stress (Takahashi-Niki *et al.*, 2004). However DJ-1 mutations are rare in PD.

Overall, these genes express proteins that are involved in one or more pathological pathways in PD including mitochondrial dysfunction, ubiquitin proteasome system dysfunction and oxidative stress (see Section 1.1.4), reinforcing their contribution to cell death in PD. Although genetic mutations have contributed greatly to the understanding of pathogenesis of PD, they do not explain the majority of sporadic PD cases. Nevertheless, research in this area is on-going and it is possible that future investigations may uncover new susceptibility genes in sporadic PD.

PARK Locus	Gene	Map Position	Inheritance
Well-validated loci/genes			
PARK1/PARK4	*SNCA*	4q21	AD
PARK2	*Parkin*	6q25.2–q27	AR
PARK6	*PINK1*	1p35-p36	AR
PARK7	*DJ-1*	1p36	AR
PARK8	*LRRK2*	12q12	AD (incomplete penetrance)
PARK9	*ATP13A2*	1p36	AR
Putative loci/genes			
PARK3	Unknown	2p13	AD
PARK5	*UCHL1*	4p14	AD
PARK10	Unknown	1p32	Not clear
PARK11	*GIGYF2*	2q36–q37	AD (incomplete penetrance)
PARK12	Unknown	Xq21-25	Not clear
PARK13	*Omi/HTRA2*	2p12	Not clear
PARK14	*PLA2G6*	22q13.1	AR
PARK15	*FBXO7*	22q12–q13	AR

Table 1.2 Genes underlying monogenic parkinsonism.

AD, autosomal dominant; AR, autosomal recessive. Table adapted from (Bekris *et al.*, 2010).

Genetic mutations account for less than 10% of the cases thus the disease is more commonly sporadic (90%; (Litvan *et al.*, 2003)). Parkin and PINK1 mutations are suggested to explain up to 50% of early onset PD cases (less than 40 years old) though this only accounts for 1-2% of total PD cases (Hardy, 2010). Therefore, some suggest that environmental agents are the main causal factor, and this is supported with the following evidence. The ability of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to induce neurochemical, pathological and clinical features similar to those of PD suggested that similar effects could be caused by other environmental neurotoxins (Di Monte *et al.*, 2002). In addition, most epidemiological studies have found increased exposure to pesticides to be associated with higher risk of PD (Gorell *et al.*, 1998; Liou *et al.*, 1997; Petrovitch *et al.*, 2002; Seidler *et al.*, 1996; Semchuk *et al.*, 1992). In fact, pesticides such as rotenone and paraquat induce loss of nigral dopaminergic neurons in rodents (Greenamyre *et al.*, 2003; Peng *et al.*, 2004). Well water consumption and living in rural areas associated with the agricultural industry have also been linked to an increased risk of PD (Gatto *et al.*, 2009; Priyadarshi *et al.*, 2001). In addition, people chronically exposed to welding fumes exhibited PD-like clinical features (Jankovic, 2005) and this was attributed to the high levels of manganese in these fumes (Jankovic, 2005). Further, metals such as iron and copper accumulate in SN and contribute to the production of hydrogen peroxide during the enzymatic oxidation of dopamine and the conversion of hydrogen peroxide to hydroxyl radicals (Dexter *et al.*, 1989b; Earle, 1968). There is a large body of epidemiological evidence of a link between exposure to toxins and PD, providing good grounds for further investigating the effects of long term exposure to toxins. Nevertheless, toxin exposure only increases the risk and does not cause PD in all exposed individuals, suggesting that some people may be more susceptible than others and this could be due to genetic factors.

In view of the evidence, the theory that a combination of genetic and environmental factors leads to PD may be a plausible possibility. Although the causal factors are still not fully understood, PD has well defined pathological hallmarks.

1.1.3 Pathology of PD

The pathological hallmarks of PD are a loss of dopaminergic neurones in the substantia nigra pars compacta (SNpc) and presence of inclusion bodies termed Lewy bodies (LBs) in surviving neurons. Inflammatory gliosis is also considered a hallmark of PD pathology (see Section 1.1.4.g).

The main site of dopaminergic neuronal loss in PD is the SNpc leading to depletion of dopamine from terminals in the striatum. The depletion of striatal dopamine causes motor symptoms of PD because the nigro-striatal pathway is a major component of the basal ganglia that controls voluntary movement. Motor symptoms of PD do not manifest until approximately 50-60% of nigral dopaminergic cell bodies have degenerated and about 70-80% decrease of striatal dopamine content is reached (Agid, 1991; Fearnley *et al.*, 1991). This is due to compensatory mechanisms, acting to maintain normal function, which include increased dopamine turnover and an increase in post-synaptic dopamine D_2 receptors in the striatum (Bezard *et al.*, 2003). As neuronal loss progressively increases, compensatory mechanisms eventually become unable to maintain normal basal ganglia function and clinical symptoms appear. Neurodegeneration also occurs in other brain regions including locus coeruleus (LC), raphe nuclei (RN), dorsal motor nucleus of the vagus (DMNV), nucleus basalis of meynert (NBM), the olfactory system and in peripheral autonomic ganglia, although to a lesser extent (Javoy-Agid *et al.*, 1984; Lang *et al.*, 1998; Zarow *et al.*, 2003). Degeneration in these areas may be responsible for the non-motor symptoms, for example anxiety and depression are linked to the loss of noradrenergic and serotonergic neurons in the LC and RN and dementia is believed to be the result of the degeneration of the cholinergic system (Calabresi *et al.*, 2006; Hanagasi *et al.*, 2005).

Neuronal degeneration is accompanied by the formation of LBs. LBs are intraneuronal proteinaceous cytoplasmic inclusions composed of various proteins includin ubiquitin and α-synuclein (Olanow *et al.*, 2004). In PD, LBs are found in the remaining neurones of SN but also in noradrenergic neurons of LC, cholinergic neurones of the DMNV and NBM and in the hypothalamus (den *et al.*, 1960; Langston *et al.*, 1978; Olanow *et al.*, 2004). The role of LBs in PD is not well understood. Recent evidence showed that clinically healthy individuals with α-

synuclein-immunoreactive LBs, incidental lewy body disease (ILBD) have reduced striatal Tyrosine hydroxylase (TH) and VMAT2 and nigal neuron loss, indicating that ILBD may be an early stage of PD (DelleDonne *et al.*, 2008). This suggests that LBs may be formed before clinical manifestation of the disease. In addition, the increase of LB numbers in the SN is suggested to be associated with less surviving dopaminergic neurons (de la Fuente-Fernandez *et al.*, 1998). These findings may suggest that LBs contribute to or at least are an indication of pathogenic load on neurones, but some suggest that LBs may be protective to neurons by means of removing potentially toxic proteins (Olanow *et al.*, 2004).

Braak *et al*. analysed brains from PD patients and asymptomatic subjects with LB pathology, with varying spread of pathology using α-synuclein immunopositive Lewy neurites and LBs as a neuropathological marker and suggested a neuropathological staging procedure describing the progress of pathology in terms of topographical extent (Braak *et al.*, 2003). According to the authors, pathology starts at the dorsal motor nucleus of the vagal nerve (stage 1) gradually progressing in an ascending fashion to reach the neocortex at stages 5-6 (Figure 1.1). The SN only starts to be affected at stage 3 but the symptomatic phase is suggested to start at stages 4-5 where severe neuronal loss occurs at the SN. This may be an explanation of the progressive nature of PD. However, this staging procedure received the criticism that it is based on the presence of α-synuclein not neuronal loss and on the assumption that pre-symptomatic patients with LBs will develop PD at a later stage (Linazasoro, 2007). In addition, other studies showed evidence of cases that do not follow the stages described by Braak *et al*. since in some cases there was α-synuclein pathology in the SN but not in the LC and DMNV (Jellinger, 2008; Parkkinen *et al.*, 2005).

LBs are clearly associated with degeneration of dopaminergic neurons. However, in PD, not all nigral neurons contain LBs (Halliday *et al.*, 1990) suggesting that there may be differences in the pathogenic processes in neurons or that some neurons may take longer to form or do not form LBs. It may also suggest a complexity in the pathogenesis leading to neurodegeneration in PD. Death of nigral dopaminergic neurones is in fact a result of complex and interrelated pathogenic mechanisms, as discussed below.

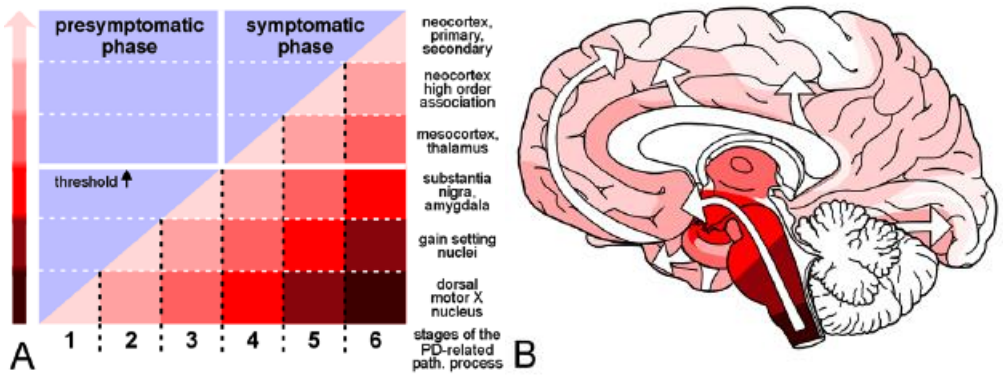

Figure 1.1 Neuropathological stages of PD.

(A,B) PD neurodegenerative pathology spreads in an ascending pathway in the brain in 6 stages (Braak *et al.*, 2004). White arrows indicate the ascending pathology process.

1.1.4 Mechanisms of cell death

The exact mechanism by which nigral dopaminergic neurons undergo degeneration is still unknown. Evidence indicates that mitochondrial dysfunction, oxidative and nitrative stress, inflammation, proteasomal system dysfunction, necrosis and apoptosis are all involved in cell death of dopaminergic neurons in PD as discussed below. There is also cumulative evidence that excitotoxicity contributes to cell death in PD, but this awaits confirmation by direct evidence from PD brains (Olanow, 2007). Nevertheless, the precise way and exact order in which these pathways lead to cell death remains to be solved.

1.1.4.a Apoptosis and necrosis

Necrosis is suggested as a possible cell death mechanism in PD (Jellinger, 2000), although strong evidence supporting this has not been found. Indirect evidence of the involvement of necrosis in dopaminergic cell death comes from the ability of dopaminergic neurotoxins to cause necrotic cell death in dopaminergic cell lines (Maruyama *et al.*, 2000; Ochu *et al.*, 1998). Necrosis is a passive form of cell death characterised by loss of ATP, cell swelling, membrane and organelle degradation and ultimately cell lysis, causing release of cell content and damage to surrounding tissue (Levin *et al.*, 1999). Conversely, apoptosis is programmed cell death, characterised by morphological changes including cell shrinkage, nuclear condensation, and DNA degradation (Majno *et al.*, 1995). There is a large body of

evidence suggesting the involvement of apoptosis as a major cell death pathway in PD pathogenesis. *In-vitro*, the dopaminergic neurotoxin 6-OHDA was found to induce apoptosis in PC-12 cells, and primary dopaminergic cells (Blum *et al.*, 2001; Blum *et al.*, 1997; Lotharius *et al.*, 1999). MPP^+ treatment also induces apoptosis in PC-12 and VM culture cells (Hartley *et al.*, 1994; Viswanath *et al.*, 2001). In addition, inhibition of apoptosis seems to prevent cell death. Pre-treatment of PC-12 cells with caspase inhibitors protected the cells from 6-OHDA induced apoptosis (Takai *et al.*, 1998). Overexpression of the anti-apoptotic protein bcl-2 also prevented 6-OHDA induced apoptosis (Offen *et al.*, 1997; Takai *et al.*, 1998).

Moreover, animal studies have also provided evidence for the possible involvement of apoptosis in PD. 6-OHDA lesioning of rats induces apoptosis of nigral dopaminergic neurons (He *et al.*, 2000; Zuch *et al.*, 2000) as demonstrated by an increase in the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining and morphological changes. MPTP treated mice also showed evidence of apoptotic cell death including DNA fragmentation, JNK activation and bax mRNA upregulation (Saporito *et al.*, 2000; Tatton *et al.*, 1997; Vila *et al.*, 2001b). Additionally, ablation of the pro-apoptotic Bax gene as well as overexpression of bcl-2 were shown to provide protection against MPTP-induced nigral dopaminergic cell death in mice (Offen *et al.*, 1997; Vila *et al.*, 2001b; Yang *et al.*, 1998).

In post-mortem studies, morphological signs of apoptosis such as chromatin condensation and irregular nuclear morphology are evident in brains of PD patients (Anglade *et al.*, 1997; Kingsbury *et al.*, 1998; Tompkins *et al.*, 1997). In addition, an increase in the expression of the apoptotic markers bax and caspase-3 was observed in SN in PD (Hartmann *et al.*, 2000; Tatton, 2000) as well as an increase in the level of the anti-apoptotic protein bcl-2 (Mogi *et al.*, 1996). In a separate study, caspase-1 and caspase-3 activity were significantly higher in the SN of PD patients compared to controls (Mogi *et al.*, 2000). Conversely, some studies failed to find evidence of apoptotic cell death in post mortem PD tissue (Jellinger, 2000; Vyas *et al.*, 1997). This, however, maybe due to the use of less sensitive classical histochemical techniques.

In summary, there is some evidence of the involvement of necrosis in PD but this has not been extensively studied. On the other hand, although some groups

failed to find evidence of apoptosis in PD, there is a large body of evidence from *in-vitro*, *in-vivo* and post mortem PD brains suggesting that cell death in PD is more likely apoptotic. The exact pathway leading to apoptosis is unknown but oxidative and nitrative stress, mitochondrial dysfunction, inflammation, UPS dysfunction and autophagy are suggested to be contributory mechanisms.

1.1.4.b Oxidative stress

Oxidative stress is implicated as a major pathogenic pathway in dopaminergic neurodegeneration. It is defined as the imbalance between production of ROS and endogenous anti-oxidant systems. Many cellular reactions such as mitochondrial oxidative respiration and dopamine metabolism require the use of molecular oxygen for catalysis and energy production. A consequence of these reactions is production of reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide, hydroxyl radicals, peroxy radicals which are all important for physiological functions (Imlay, 2003). However, overproduction of ROS can lead to damage and destruction of DNA, proteins and membranes (Loh *et al.*, 2006). Interestingly, oxidative stress seems to increase with age as there is evidence of more oxidized proteins, including carboxyls and nitro-protein adducts in ageing brains (Cardoso *et al.*, 2005; Mariani *et al.*, 2005). Elderly brains also have higher levels of 8-hydroxy-2-deoxy-guanosine (8-HDG), a marker of oxidized DNA, in both nuclear and mitochondrial DNA (Mariani *et al.*, 2005). This may be a predisposing factor to neurodegenerative disease involving oxidative stress. In fact, dopaminergic neurons may be particularly subjected to oxidative stress due to production of free radicals during dopamine auto-oxidation or metabolism (Lotharius *et al.*, 2002; Perez *et al.*, 2004). Dopamine has a tendency to auto-oxidize into dopamine-quinone species, superoxide radicals, and hydrogen peroxide (Graham *et al.*, 1978). These molecules are either highly reactive molecules or can quickly generate free radicals. However, dopamine metabolism alone cannot explain neurodegeneration in PD since other non-dopaminergic neuronal types also undergo degeneration (Ahlskog, 2005).

Direct evidence of oxidative stress was found in post mortem PD brains where there was an increase in protein carbonyls, proteins with oxidised amino acids in the SN (Alam *et al.*, 1997; Floor *et al.*, 1998). Increased levels of lipid peroxidation markers including malondialdehyde, cholesterol lipid hydroperoxides and 4-hydroxy-2-nonenal have also been reported in the SN of PD brains (Dexter *et al.*,

1989a; Dexter *et al.*, 1994; Yoritaka *et al.*, 1996; Zhang *et al.*, 1999). In addition, 8-HDG is shown to be increased in the SN and striatum of PD patients (Alam *et al.*, 1997; Zhang *et al.*, 1999).

Endogenous antioxidants such as glutathione and vitamin E as well as antioxidant enzymes such as superoxide dismutase, catalase and gluthathione peroxidase exist in cells in order to balance the oxidative mechanisms. For example, superoxide dismutase can decrease oxidative stress by metabolising the conversion of superoxide to H_2O_2 which is then transformed into water and oxygen by catalase (Lin *et al.*, 2005). Decreased levels of endogenous antioxidants contribute to the oxidative stress in cells. In fact, levels of glutathione and its oxidized product, which act as antioxidants, were found to be decreased in SN of PD brains compared to control (Pearce *et al.*, 1997; Sofic *et al.*, 1992). Levels of glutathione are also decreased in individualss with ILBD, which may be indicative of pre-symptomatic PD, to a level similar to that in advanced PD, suggesting that oxidative stress is an early event in the pathogenesis of PD (Dexter *et al.*, 1994).

In conclusion, there is considerable evidence to suggest oxidative stress as a pathogenic pathway in dopaminergic cell death. Oxidative stress, in turn, may precipitate other cell death pathways. It can affect performance of the ubiquitin-proteasome system (UPS) by oxidation of proteins of the proteasome (Reinheckel *et al.*, 2000; Shamoto-Nagai *et al.*, 2003) and may increase the load on the UPS system by the accumulation of oxidatively damaged proteins within cells. There is also evidence suggesting a link between mitochondrial dysfunction and oxidative stress. For instance, mice deficient in superoxide dismutase or glutathione peroxidase show higher vulnerability to the mitochondrial toxin MPTP (Klivenyi *et al.*, 2000). Furthermore, ROS not only contribute to oxidative stress but also react with nitric oxide (NO) leading to the accumulation of reactive nitrogen species (RNS) which contribute to nitrative stress, another pathway implicated in cell death in PD.

1.1.4.c Nitrative stress

Nitrative stress occurs when there is an imbalance between (RNS) and the anti-oxidant defence mechanisms. Nitrative stress results from excessive formation of the free radical NO which reacts with ROS leading to the accumulation of RNS such as peroxynitrite (EstÉVez *et al.*, 2002). Peroxynitrite can directly cause oxidative

damage to several molecules or indirectly by forming other reactive radicals including hydroxyl radicals (EstÉVez *et al.*, 2002). Peroxynitrite can then oxidise or nitrate many vital macromolecules including tyrosine residues, thiols and DNA leading to DNA strand breaks, disruption of protein integrity and enzyme dysfunction (Beckman *et al.*, 1996; Kennedy *et al.*, 1997).

Nitrative stress is suggested to be involved in the pathogenesis of PD and this is demonstrated by the evidence of accumulation of nitrated α-synuclein in LBs of post-mortem PD brains (Giasson *et al.*, 2000; Good *et al.*, 1998). There is also evidence of nitrated α-synuclein in the SN of MPTP-treated mice (Schapira *et al.*, 2010). In addition, 3-nitrotyrosine (3-NT), which results from protein nitration, was found to be increased in the ventral midbrain and striatum of MPTP-treated mice (Pennathur *et al.*, 1999). Further, presence of inducible nitric oxide synthase (iNOS) is evident in the SN of PD brains (Hunot *et al.*, 1996). iNOS produces high amounts of NO (Luo *et al.*, 2000) and is induced in the brain in pathological situations such as inflammation, infection and ischemia and also in the aging brain (Licinio *et al.*, 1999). In PD, iNOS is induced in glial cells, particularly in microglia (Hunot *et al.*, 1996; Knott *et al.*, 2000b). iNOS is induced in microglia following Lipopolysaccharide (LPS) and MPTP treatment in rodents (Arimoto *et al.*, 2003; Dehmer *et al.*, 2000; Iravani *et al.*, 2002; Iravani *et al.*, 2005; Liberatore *et al.*, 1999).

Moreover, the tyrosine-nitration of mitochondrial complex-I resulting in its inhibition is suggested to be involved in the pathogenesis of PD (Murray *et al.*, 2003). In fact, nitration of mitochondrial complex-I has been associated with a decrease in glutathione levels (Bolanos *et al.*, 1996). Further, nitration of striatal TH, enzyme involved in synthesis of dopamine, in MPTP-treated mice resulted in enzyme inactivation and consequently failure of dopamine synthesis (Ara *et al.*, 1998). There is further indirect evidence of the involvement of nitrative stress in PD pathology as, for instance, nitric oxide synthase (NOS) inhibitors were shown to protect against LPS induced nigral cell death in rats (Iravani *et al.*, 2002). iNOS KO mice are also protected against MPTP toxicity (Liberatore *et al.*, 1999).

In summary, there is strong evidence to suggest the implication of nitrative stress as a pathogenic mechanism in PD. However, it is not fully clear how nitrative stress is linked to the cascade of events leading to cell death in PD.

1.1.4.d Mitochondrial dysfunction

Mitochondrial dysfunction has been implicated as one major pathogenic pathway in PD. Mitochondria are the energy manufacturing entity in the cell and their dysfunction results in energy deficiency and activation of cell death pathways. Understanding the role of mitochondrial dysfunction in the underlying pathology of PD stems from studies of the MPTP-induced Parkinson-like syndrome in man (Langston *et al.*, 1983) and non-human primates (Burns *et al.*, 1983). MPTP leads to dopaminergic cell death in humans via inhibition of mitochondrial complex I by inhibiting the enzyme nicotinamide dehydrogenase (NADH) (Singer *et al.*, 1987) and by generating free radicals (Cleeter *et al.*, 1992). Inhibition of NADH, in turn leads to superoxide production and lipid peroxidation (Hasegawa *et al.*, 1990a; Sriram *et al.*, 1997). This suggested that mitochondrial dysfunction may contribute to neurodegeneration in PD. This concept is supported by evidence that other toxins which cause complex I inhibition such as rotenone, also lead to selective nigral dopaminergic cell death in rats and formation of inclusion bodies (Sherer *et al.*, 2003b).

The involvement of mitochondrial dysfunction in PD is reinforced by evidence from post-mortem studies in idiopathic PD brains where there is a 30-40% decrease in mitochondrial complex I activity in SN (Janetzky *et al.*, 1994; Mann *et al.*, 1994; Schapira *et al.*, 1990). There is also a decrease in complex I proteins in the SN of individuals with PD (Mizuno *et al.*, 1989). Specificity of complex I deficiency was demonstrated by reduced histochemical staining of complex I subunits in PD but not other respiratory chain complexes (Hattori *et al.*, 1991) in addition to the finding that it is the only complex affected by endogenous oxidative damage and reduced structural stability in PD (Keeney *et al.*, 2006). Recently, mitochondrial complex I impairment was observed in the frontal cortex of PD patients (Parker Jr *et al.*, 2008).

Additionally, complex I activity was decreased in platelet mitochondria from PD patients (Haas *et al.*, 1995). In fact, transfer of mitochondrial DNA (mtDNA) from platelets of PD patients to cells lacking mtDNA resulted in recipient cybrid cells with complex I deficiency, suggesting a genetic base to mitochondrial dysfunction (Gu *et al.*, 1998; Swerdlow *et al.*, 1996). In fact, the cybrid cells showed higher ROS production and increased susceptibility to MPP^+ (Swerdlow *et al.*, 1996). An increase in deleted nigral mtDNA was observed in post-mortem PD brains

(Bender *et al.*, 2006). Furthermore, the fact that genes causing familial PD encode mitochondrial proteins such as PINK1 and DJ-1 suggests that mutations in these genes may lead to PD via vulnerability or dysfunction of mitochondrial proteins (Schapira, 2008a). In support of this concept, PINK1 KO mice displayed impaired mitochondrial function, and higher sensitivity to oxidants (Gautier *et al.*, 2008) and DJ-1 mutations are associated with higher sensitivity to oxidative stress and greater nigro-striatal neurodegeneration following MPTP treatment (Kim *et al.*, 2005).

Mitochondrial dysfunction, in turn, may precipitate other pathological pathways. Inhibition of complex I activity by MPTP *in-vitro* leads to increased production of peroxide radicals hence contributing to oxidative stress (Hasegawa *et al.*, 1990b). Also, defective mitochondrial complex I leads to higher vulnerability of cells to apoptosis due to the generation of free radicals and sensitisation of cells to the pro-apoptotic protein Bax (Perier *et al.*, 2005).

In view of the evidence, mitochondrial dysfunction is therefore an evident pathological mechanism in PD and may exacerbate other pathological pathways thought to be involved in dopaminergic cell death. However, further work is required to establish the exact mechanism leading to cell death.

1.1.4.e Ubiquitin proteasomal system impairment

Failure of the ubiquitin-proteasome system (UPS) is also suggested to be involved in neuronal degeneration in PD. The UPS is a protein degradation system for unwanted proteins including mis-located, mis-folded, mutant or damaged proteins (Ciechanover, 1998). Proteins are first tagged with ubiquitin forming polyubiquitin-protein conjugates (Hochstrasser, 1996; Pickart, 2000). These tagged proteins are then identified and metabolised by the proteasomes (Brodsky *et al.*, 1997; Hochstrasser, 1996; Pickart, 1997). Failure of this system leads to accumulation of unwanted proteins within the neuron and may result in or contribute to cell death. In fact, proteasome inhibition in neuronal cells *in-vitro* was found to be associated with the accumulation of ubiquitinated proteins, a pro-inflammatory response and apoptotic cell death (Canu *et al.*, 2000; Rockwell *et al.*, 2000).

The link between failure of the UPS and PD was supported by the finding that LBs in PD accumulate ubiquitinated proteins involved in the UPS such as parkin, ubiquitin, and UCHL-1 (Olanow *et al.*, 2004) and that mutations in genes involved in

protein processing and degradation including parkin, UCHL-1 and ubiquitin have been found in familial cases of PD (Bekris *et al.*, 2010). In addition, systemic administration of proteasomal system inhibitors in rodents resulted in nigral dopaminergic degeneration and formation of α-synuclein and ubiquitin positive inclusions (Bukhatwa *et al.*, 2009; McNaught *et al.*, 2004; Sun *et al.*, 2006). Direct evidence from post-mortem brains of PD patients has immerged to support a role of the failure of the UPS in the pathogenesis of PD. A reduction of proteasomal α subunit expression was observed in SNpc dopaminergic neurons of sporadic PD patients compared to control (Bukhatwa *et al.*, 2010b; St. P. McNaught *et al.*, 2003). Further, proteasomal enzymatic activity was found to be reduced in SN from PD patients compared to control (Bukhatwa *et al.*, 2010a; Olanow *et al.*, 2006).

In summary, there is evidence of impaired UPS function in PD which may result in accumulation and aggregation of damaged and misfolded proteins ultimately causing cell death. This protein load on cells can also be caused by dysfunction of lysosomal degradation.

1.1.4.f Autophagy

Autophagy is normally part of the homeostatic system in cells, it is a mechanism for the degradation of unwanted proteins including long-lived proteins, aggregate-prone proteins and cytoplasmic organelles (Rubinsztein, 2006). There are three types of autophagy, macroautophagy; in which proteins are sequestered into autophagosomes which then fuse with lysosomes, microautophagy; in which proteins are sequestered directly into lysosomes and chaperon mediated autophagy (CMA); in which target proteins are recognised by heat-shock cognate 70 (Hsc70), followed by transportation directly into the lysosomes (Yang *et al.*, 2010). A number of studies have shown that CMA is involved in alpha-synuclein degradation (Cuervo *et al.*, 2004; Vogiatzi *et al.*, 2008; Webb *et al.*, 2003). In fact, inhibition of autophagy leads to the accumulation of mono-ubiquinated alpha-synuclein, which can trigger aggregation (Rott *et al.*, 2008). In addition, A53T and A30P mutants of alpha-synuclein, which cause familial PD, were found to inhibit autophagy (Cuervo *et al.*, 2004) suggesting that these mutations may induce aggregation of alpha-synuclein through inhibition of autophagy. Besides, inhibition of autophagy resulted in higher apoptosis in cell lines transfected with A53T or A30P mutants of alpha-synuclein than control cells (Yang *et al.*, 2009). Macroautophagy is involved in the

degradation of dysfunctional mitochondria. Importantly, Parkin was found to contribute to mitochondrial turnover by facilitating macroautophagy of dysfunctional mitochondria (Narendra *et al.*, 2008). Therefore, Parkin mutation, present in familial PD, may lead to impairment of mitochondrial turnover and thereby contribute to cell death. Deletion of PINK1 gene -involved in the regulation of mitochondrial function and mutations of which are present in some familial PD cases- is shown to induce autophagy of mitochondria (Dagda *et al.*, 2009). This suggests that autophagy is implicated in mitochondrial turnover and failure of this degradation mechanism may be linked to cell death in PD. In conclusion, impairment of autophagy may lead to neuronal cell death via accumulation of aggregated proteins and dysfunctional mitochondria.

On the other hand, some suggest that excessive activation of autophagy may also be pathogenic (Bredesen *et al.*, 2006). In support of this, *in-vitro* treatment with MPP^+ is shown to increase autophagic vacuoles (Zhu *et al.*, 2007) and overexpression of A53T mutant of α-synuclein leads to autophagic cell death in PC-12 cells and nigro-striatal system (Kirik *et al.*, 2002; Stefanis *et al.*, 2001; Xilouri *et al.*, 2009; Yang *et al.*, 2009). In PD there is evidence of abnormal levels of autophagic vacuoles compared to normal brains (Anglade *et al.*, 1997; Zhu *et al.*, 2003). However, this may be due to over activation of autophagy but it may also be due to inability of cells to clear these vacuoles.

In light of the evidence, suboptimal activation or over-activation of autophagy may have detrimental effects on neurons. There is cumulative evidence that disregulation of autophagy may contribute to cell death in PD, but solid direct evidence supporting this is still lacking.

1.1.4.g Inflammation

There is considerable evidence of an inflammatory involvement in the pathology of PD. Cytokines such as TNF-α, IL-1β and IL-6 are expressed at high levels in SN and CSF of PD patients (Mogi *et al.*, 1994; Nagatsu *et al.*, 2000). These cytokines are thought to be responsible for many pathological and clinical manifestations of peripheral inflammatory diseases including rheumatoid arthritis and inflammatory bowel syndrome. In fact, glial cells expressing IL-1β and/or TNF-α were found in SN of PD patients (Boka *et al.*, 1994; Hunot *et al.*, 1999).

Furthermore, in post mortem brains of PD patients there is evidence of activation of microglia (McGeer *et al.*, 1988). Activated microglia are also seen in SN of humans exposed to MPTP (Langston *et al.*, 1999). Microglia are the resident immunocompetent phagocytic cells of the central nervous system which become activated in pathological events such as inflammation, infection and ischemia (Wojtera *et al.*, 2005). Activated microglia up-regulate cell surface receptors such as the macrophage antigen complex-I (MAC-1) and secrete pro-inflammatory cytokines such as IL-1β, tumor necrosis factor α (TNF-α), and NO, the overproduction of which can be neurotoxic (Nakamura, 2002). On the other hand, moderately activated microglia can play a protective role, where they scavenge neurotoxins, engulf dead cells and debris and release trophic factors such as brain-derived neurotrophic factor (Aloisi, 1999; Aloisi *et al.*, 1999; Batchelor *et al.*, 1999; Nakamura, 2002). Whether microglial activation protects or exacerbates neuronal loss remains debated (Hirsch *et al.*, 2003) but there is a vast array of evidence supporting a toxic role of activated microglia and a role of inflammation in the degenerative process as discussed hereafter.

Activated microglia in the SN in PD have been reported to express pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1, interferon IFN-γ (Boka *et al.*, 1994; Mogi *et al.*, 1994), iNOS (Nagatsu *et al.*, 2000) and cyclooxygenase-2 (COX-2) (Knott *et al.*, 2000a). The pro-inflammatory cytokines TNF-α and IL-1 can trigger direct toxicity in neurons (Allan *et al.*, 2001; Clarke *et al.*, 2002) and can also potentiate an ongoing inflammatory response by enhancing microglial NO production (Hunot *et al.*, 1999; Possel *et al.*, 2000). Further, activated microglia release ROS and NO (Dringen, 2005; Hunot *et al.*, 1996) in order to kill pathogens but these are also harmful to neurons and contribute to oxidative and nitrative stress in the brain.

Further evidence that microglial activation can lead to neurodegeneration comes from studies of the lippopolysaccharide (LPS) model of PD, where nigral injection of LPS causes microglia and astrocyte activation and degeneration of dopaminergic neurons (Castano *et al.*, 1998; Herrera *et al.*, 2000; Iravani *et al.*, 2005). LPS is a potent inducer of inflammation in the CNS (Goralski *et al.*, 2004) and leads to dopaminergic cell death indirectly via activation of glial cells. This is supported by evidence from *in-vitro* studies showing that, in the absence of

microglia, LPS does not cause dopaminergic cell loss (Iravani *et al.*, 2005). However, in mixed neuron-glia cultures and neuron cultures with microglial-conditioned media, LPS leads to neuronal death (Gao *et al.*, 2002; Taylor *et al.*, 2003). In addition, inhibition of iNOS attenuated LPS toxicity to neurons (Iravani *et al.*, 2005). Besides LPS; MPTP, rotenone and 6-OHDA animal models of nigral cell death in PD also display microglial activation in the SN (Cicchetti *et al.*, 2002; Czlonkowska *et al.*, 1996; Sherer *et al.*, 2003a). Whether inflammation is a primary event or secondary to neurodegeneration is still not known, though some evidence may suggest that inflammation is rather a precipitating factor. For instance, in the rotenone model, microglial activation is seen before degeneration of dopaminergic neurones (Sherer *et al.*, 2003a). In contrast, other evidence supports the possibility that it is a secondary event since humans exposed to MPTP show activated microglia 16 years after the last drug exposure (Langston *et al.*, 1999). Persistent microglial activation is also seen in primates years after MPTP treatment (Barcia *et al.*, 2004; Langston *et al.*, 1999).

Astrocytes, another type of glia present in the SN are also involved in the inflammatory reaction associated with PD. Astrocytes protect neurons from cell death by releasing neurotrophic factors such as GDNF and BDNF and by increasing activity of glutathione reductase (Chen *et al.*, 2006; Ishida *et al.*, 2006; Knott *et al.*, 2002). In contrast, activated astrocytes may contribute to neuronal death as they were shown to release the cytotoxic compounds NO, glutamate and H_2O_2 in VM cultures (McNaught *et al.*, 1999b). Activated astroglia show an increase in the expression of glial fibrillary acidic protein (GFAP), enlarged cell body and processes (Eddleston *et al.*, 1993). In the SN of post-mortem PD brains there is evidence of astrocytes activation in some but not all cases of PD (Forno *et al.*, 1992). Activated astrocytes were also found in post-mortem brains of MPTP-exposed individuals (Langston *et al.*, 1999). In addition, astrocytic activation is also seen in animal models of PD such as MPTP treated monkeys (Kohutnicka *et al.*, 1998; Liberatore *et al.*, 1999) 6-OHDA lesioned rats (Gordon *et al.*, 1997; Pasinetti *et al.*, 1999; Rodriguez *et al.*, 2001) and LPS lesioned rats (Castano *et al.*, 1998; Herrera *et al.*, 2000).

Furthermore, evidence suggests that anti-inflammatory drugs may attenuate or prevent dopaminergic neurodegeneration indicating that the underlying mechanism of cell death is inflammatory in nature. One epidemiological study found that NSAID use is associated with a 45% decrease in PD risk (Chen *et al.*, 2003a). However, a later study found that NSAIDs use was associated with lower risk of PD in men but a higher risk in women (Hernan *et al.*, 2006). Interestingly, a lower risk of Alzheimer's disease in NSAID users, or those suffering from diseases in which anti-inflammatory agents are routinely taken has been confirmed in more than 20 studies (McGeer *et al.*, 2007).

Many investigations of anti-inflammatory agents have emerged with some promising *in-vivo* results. The tetracycline antibiotic derivative with anti-inflammatory properties, minocycline, prevented nigral dopaminergic cell loss in the MPTP and 6-OHDA models (Du *et al.*, 2001; He *et al.*, 2001; Wu *et al.*, 2002) and prevented iNOS up-regulation (Wu *et al.*, 2002). The anti-inflammatory glucocorticoid, dexamethasone, was shown to partially inhibit microglial activation, decrease release of pro-inflammatory cytokines and NO and to protect against dopaminergic neurodegeneration caused by MPTP and intra-nigral LPS (Castano *et al.*, 2002). A peroxisome proliferator-activated receptor-γ (PPARγ), pioglitazone has also shown neuroprotection against MPTP toxicity in mice (Breidert *et al.*, 2002). The nonselective COX-2 inhibitor sodium salicylate has also shown neuroprotective activity in-vivo (Gao *et al.*, 2003; Sanchez-Pernaute *et al.*, 2004). However, most anti-inflammatory agents tested have not reached the clinical stage and whether they will have an effect on progress of PD remains to be determined. A recent phase II futility trial with minocycline in early onset PD patients recommended phase III trials to be conducted in order to determine whether minocycline would affect progression of the disease since it was well tolerated and improved the UPDRS score (investigators, 2008).

In summary, there is a large body of evidence supporting the involvement of inflammation in PD pathogenesis. However, it is still not known whether inflammation precedes or occurs as a result of neuronal degeneration. Although clinical trials have not yet determined whether anti-inflammatory agents have neuroprotective activity in PD patients, pre-clinical data indicates that an anti-inflammatory agent may have protective effects. Inflammation is an important

process in the brain, and it seems that if regulated properly, it is beneficial to neurons in case of injury by eliminating damaged neurons and promoting survival of those that have a possibility to survive (Marchetti *et al.*, 2005). However, inflammation can become a chronic uncontrolled process. Therefore, studying regulatory factors of inflammation may help elucidate agents that would maintain protective effects but not deleterious ones. One such factor may be the protein osteopontin (OPN), a protein released in inflammatory processes and promotes tissue repair and cell survival. This may be a good candidate to investigate as a potential neuroprotective treatment for PD since it was recently shown to be expressed in the SN and its levels reduced in remaining dopaminergic neurons in PD (Iczkiewicz *et al.*, 2006). The properties of OPN and its potential for neuroprotection are explained in the following section and its effects in models of nigral cell death form the basis of this thesis.

1.2 OPN: a potential neuroprotective agent

OPN, a phosphorylated glycoprotein first isolated from transformed cells as a 60kDa protein in 1979 (Senger *et al.*, 1979). Subsequently, it was discovered by separate groups as a transformation association gene 2ar (Smith *et al.*, 1987) and as a cytokine released by activated macrophages and lymphocytes and was named early lymphocyte activation gene 1 (Eta-1) (Patarca *et al.*, 1989a). Interestingly, as discussed below, OPN is shown to regulate inflammation, reduce nitrative and possibly oxidative stress, inhibit apoptosis and to be present in the brain. Therefore, this protein may have multifactorial protective effects and will be investigated as a potential protective agent against the degeneration of dopaminergic neurones in PD. Structure, functions, and receptors mediating effects of OPN are described below.

1.3 OPN structure

OPN belongs to a family of proteins, all being expressed in bone or dentin and having the ability to bind at least one receptor from the integrin family, together termed SIBLING (small integrin binding ligand N-linked glycoproteins) (Fisher *et al.*, 2001). Proteins of this family are coded by genes situated on chromosome 4 in man. They act as signal transducers to promote cell adhesion, motility and survival but each protein has specific roles (Bellahcene *et al.*, 2008). OPN is expressed as a 34 kDa nascent protein by a single copy gene and is subject to substantial post–translational modifications explaining the varying reported size (44 to 75 kDa) (Kasugai *et al.*, 1991; Senger *et al.*, 1989; Wrana *et al.*, 1991). It consists of 264 to 301 amino acids depending on the species. Moderate levels of sequence conservation (40%) between five species (rat, mouse, human, pig, cow) exist in the -NH_2 and -COOH terminal regions (Butler, 1995) suggesting functional importance of these sequences. The amino acid serine shows the highest level of conservation (24% in all 6 species are conserved) and glutamic acid shows a relatively high level of conservation (17%), indicating the importance of sequences subject to kinase activities and phosphorylation (Butler, 1995). In fact, OPN undergoes heavy post-translational modifications which may regulate its function as explained henceforth.

OPN is acidic in character, being rich in aspartic acid and glutamic acid (Butler, 1995; Chen *et al.*, 1992; Prince, 1989). It contains N- and O-glycosylation sites in addition to multiple consensus sites for possible Ser and Thr phosphorylation

(Christensen *et al.*, 2005; Senger *et al.*, 1994; Sørensen *et al.*, 1995) suggesting possibility of tissue specific isoforms of OPN. Conservation and juxtaposition of several serine and glutamic acid residues suggests that phosphorylation of the protein is important to its function (Butler, 1995). This is supported by reports of different functions of the protein to be related to different phosphorylation patterns (Nemir *et al.*, 1989; Singh *et al.*, 1990). In addtion, the degree of phosphorylation affects the effectiveness of mineralization (Gericke *et al.*, 2005) and mineral induction (Salih *et al.*, 2002).

OPN contains a conserved polyaspartic acid motif which is suggested to bind the protein to hydroxyapatite and calcium ions (Butler, 1995; Gorski, 1992), an RGD (arg-gly-asp) integrin binding domain (Butler, 1995; Oldberg *et al.*, 1986) and a second integrin binding sequence SVVYGLR (ser-val-val-tyr-glu-leu-arg) (Yokasaki *et al.*, 2000) (Figure 1.2). These are the main identified receptor binding sites of the protein. The region consisting of about 50 amino acids bracketing the RGD sequence exhibits the highest level of sequence conservation, suggesting importance of this sequence to the function of the protein (Denhardt *et al.*, 1993). OPN also contains two consensus heparin binding domains (Prince, 1989). Besides integrins, OPN also binds to the CD44 receptor but the exact binding domain has not been determined (Weber *et al.*, 1996a). Additionally, the protein is cleaved by thrombin at the RS (arg-ser) site, 7 amino acids away from the RGD site (O'Regan *et al.*, 1999; Senger *et al.*, 1994). Thus thrombin cleavage may regulate the interaction with integrin receptors. This concept is supported by evidence showing that thrombin cleavage enhances actions of OPN such as cell attachement and spreading (Senger *et al.*, 1994) and that it is necessary for interaction with integrins $\alpha_9\beta_1$ and $\alpha_4\beta_1$ (Bayless *et al.*, 2001; Smith *et al.*, 1996; Yokasaki *et al.*, 2000). OPN is also a substrate for cleavage by matrix metalloproteinase 3 (MMP-3) and MMP-7 at a limited number of sites including the GL sequence immediately before thrombin cleavage site (Agnihotri *et al.*, 2001). Cleavage by MMPs also enhances OPN mediated cell adhesion and migration (Agnihotri *et al.*, 2001). The COOH terminal half of the protein after thrombin cleavage contains a non RGD cell attachment site, identity and significance of which are unknown (van Dijk *et al.*, 1993). This attachement, however, was inhibited by RGD peptides and anti-$\alpha_v\beta_3$ antibody (van Dijk *et al.*, 1993). The secondary structure of OPN (Figure 1.2b) was predicted to

contain eight α-helices and six segments of β-sheets (Denhardt *et al.*, 1993) but the full structure is yet to be solved. In summary, OPN can bind many receptors and is subject to extensive post translational modifications that may influence its function and receptor-binding. This may explain the multiple actions OPN is reported to have.

a

MRIAVICFCLLGITCAIPVKQADSGSSEEKQLYNKYPDAVATWLN
PDPSQKQNLLAPQTLPSKSNESHDHMDDMDDEDDDDHVDSQDSID
SNDSDDVDDTDDSHQSDESHHSDESDELVTDFPTDLPATEVFTPV
VPTVDTYDGRGDSVVYGLRSKSKKFRRPDIQYPDATDEDITSHME
SEELNGAYKAIPVAQDLNAPSDWDSRGKDSYETSQLDDQSAETHS
HKQSRLYKRKANDESNEHSDVIDSQELSKVSREFHSHEFHSHEDM
LVVDPKSKEEDKHLKFRISHELDSASSEVN

1 2 3 Thrombin cleavage site 4 5

b

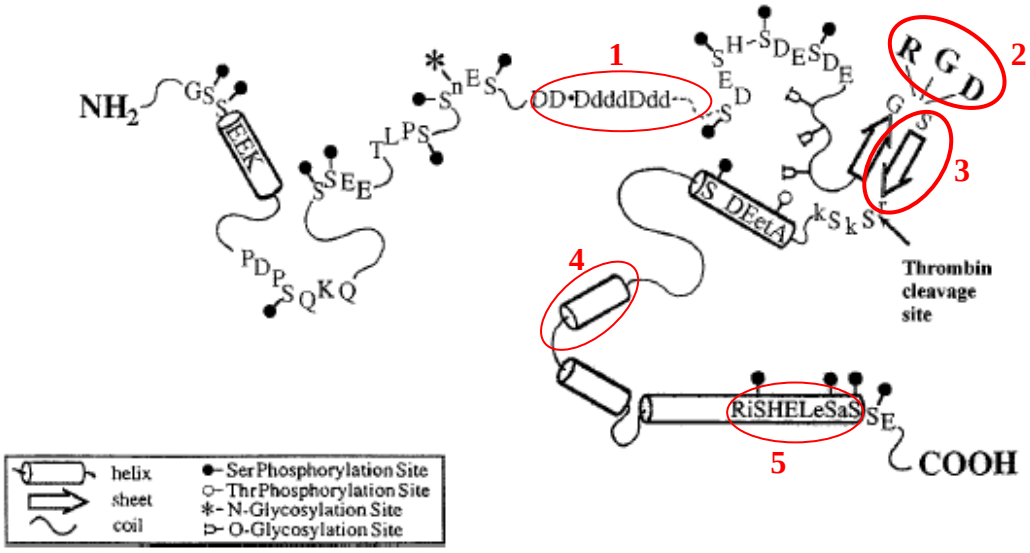

Figure 1.2 OPN predicted structure and the main functional domains.

(a) Amino acid sequence of homo sapiens OPN, Pubmed accession number, AAC28619. (b) Mammalian OPN schematic structure predicted from amino acid sequence and secondary structure prediction methods. Predicted and demonstrated post-translational modifications sites are shown on the structure. OPN is cleaved by thrombin into two equivalent size fragments; the N-terminal contains hydroxyappatite (1) and integrin binding sites RGD; (2) and SVVYGLR; (3), and the C-terminal fragment contains consensus calcium; (4) and heparin; (5) binding domains. Adapted from Sodek *et al.*, 2000.

1.4 OPN function

OPN is involved in cell adhesion and migration by receptor interaction, leading to downstream signalling events that include calcium mobilization, protein phosphorylation, and regulation of gene expression (Denhardt *et al.*, 1995). OPN is expressed in many tissues where it is reported to play different roles as discussed hereafter. The link between functions of OPN in different tissues is not very clear but overall it seems to exert effects related to its ability to bind calcium, regulate inflammation and support cell survival as summarised below.

1.4.1 OPN in bone, kidney, heart and cancer

OPN is one of the major non-collagenous proteins in bone and is secreted by osteoclasts, osteoblasts, osteocytes and chondrocytes (Denhardt *et al.*, 1993). In its phosphorylated form, OPN plays a critical role in remodelling of mature bones and negatively regulates bone calcification (Denhardt *et al.*, 2001; Sodek *et al.*, 2000). OPN null mice exhibit significantly less bone loss following ovariectomy compared to wild type controls (Yoshitake *et al.*, 1999) suggesting a role of OPN in bone resorption. It also promotes attachment of bone cells to bone matrices (Butler, 1995). In addition, OPN may play a role in regulating calcium phosphate crystal deposition. Due to the abundance and distribution of aspartate and phosphorylated serine residues, it binds strongly to calcium phosphate crystals in mineralized tissues and inhibits crystal growth (Jono *et al.*, 2000; Sodek *et al.*, 2000).

In the kidney, OPN is synthesised and secreted into urine by epithelial cells (Hudkins *et al.*, 1999; Kleinman *et al.*, 1995). OPN is suggested to inhibit renal stone formation as it was shown to reduce growth and aggregation of calcium oxalate crystals *in-vitro* (Asplin *et al.*, 1998; Shiraga *et al.*, 1992). OPN can also directly inhibit binding of calcium oxalate crystals to renal epithelial cells in culture (Lieske *et al.*, 1995). In addition, OPN null mice but not wild type mice develop significant intratubular deposition of calcium oxalate after induced hyperoxaluria (Wesson *et al.*, 2003). In the same study, in wild type mice, absence of calcium oxalate deposition was accompanied by up-regulation of OPN expression suggesting that OPN was expressed to protect against calcium oxalate deposition. Additionally, OPN is upregulated in renal injury in a pattern that correlates with monocyte

infiltration (Padanilam *et al.*, 1996; Pichler *et al.*, 1994). This suggests an involvement of OPN in the inflammatory response to kidney injury.

OPN is not expressed in normal cardiac muscle tissue, but it is thought to promote tissue repair after inflammation by inducing myocardial fibrosis and remodelling (Liaw *et al.*, 1998b; Murry *et al.*, 1994a; Trueblood *et al.*, 2001). Its presence has been detected in association with injured tissue in disease states such as dilated cardiomyopathy, hypertrophic cardiomyopathy, and ventricular tachycardia. In atherosclerosis, OPN is expressed in plaques in smooth muscle cells, epithelial cells and infiltrating macrophages (Giachelli *et al.*, 1995).

OPN is implicated in many cancers including breast, colon, prostate, osteosarcomas and melanomas (Furger *et al.*, 2001; Weber, 2001). It is thought to contribute to metastasis formation by reducing nitrative and oxidative stress produced by host macrophages (Denhardt *et al.*, 1994; Feng *et al.*, 1995). In support of this, down-regulating OPN has been shown to increase tumour sensitivity to macrophage cytotoxicity (Gardner *et al.*, 1994). In addition, its anti-apoptotic properties may contribute to anchorage independent tumour growth (Thalmann *et al.*, 1999). Investigations using OPN null mice showed conflicting results. Some studies showed that OPN does not have an effect on tumourgenesis (Chen *et al.*, 2003b; Feng *et al.*, 2000). In contrast, other studies reported that OPN secreted by the tumour promotes its growth whereas OPN secreted by macrophages causes macrophage chemo-attraction and contributes to the host's tumour reducing actions (Crawford *et al.*, 1998). The varying results may be due to the difference in tumour cell type. Different actions of OPN released from various cell types suggest that OPN's function may be regulated at the post-translational level. Differential actions of OPN in host and tumour cells, taken together with constitutive OPN expression in many other normal tissues suggest that OPN expression does not necessarily confer a tumorigenic phenotype.

1.4.2 OPN in immunity, inflammation and tissue repair

There is a large body of evidence for the involvement of OPN in the immune system though the exact role is not yet clear.

OPN is reported to induce activation and proliferation of immune cells and to play a role in different immune responses. OPN is highly expressed by activated macrophages, lymphocytes and NK cells as well as macrophage and NK-derived cells (O'Regan *et al.*, 2000). OPN expression correlates with presence of these cells in inflammatory processes and declines as inflammatory infiltrates recede. It plays a role in the migration and adhesion of T cells and macrophages and co-stimulates T cell proliferation *in-vitro* (Giachelli *et al.*, 1998; Kawashima *et al.*, 1999; Murry *et al.*, 1994b; O'Regan *et al.*, 1999; Patarca *et al.*, 1989b). OPN plays a role in cell mediated immunity where it supports a Th1 immune response and induces T cells and macrophages in culture to express Th1 but not Th2 cytokines (Ashkar *et al.*, 2000). *In-vivo*, OPN null mice have an impaired Th1 immune response and are more vulnerable to infection by Herpes simplex and Listeria monocytogenes than their wild type controls (Ashkar *et al.*, 2000). However, in a different infection model, both wild type and OPN null animals had comparable responses to antibody challenge after inducing anti-globular basement membrane nephritis (Bonvini *et al.*, 2000). Therefore, OPN may be involved in some but not all cellular immune reactions. In addition, OPN is expressed in human and mouse granulomatous responses (Ashkar *et al.*, 2000; Nau *et al.*, 1997; O'Regan *et al.*, 1999). Studies in OPN null mice showed that absence of OPN makes infectious cells proliferate more aggressively in granulomas (Nau *et al.*, 1999). In humans with a defective IFN-γ receptor 1, OPN expression in mycobacterial granuloma is impaired but also the infection has a more severe course (Nau *et al.*, 2000). Moreover, OPN may also have a role in humoral immune responses since it activates B lymphocytes (Weber *et al.*, 1996b) and induces them to express IgG and IgM (Lampe *et al.*, 1991; Prud'Homme *et al.*, 1983). The above evidence suggests a role for OPN in humoral, cell mediated as well as granulomatous immunity.

Additionally, OPN is suggested to regulate inflammatory cell function. OPN's production is induced by mediators which are expressed in the early stages of inflammatory response including tumour necrosis factor TNFα, vitamin D3 (Denhardt *et al.*, 1993; Patarca *et al.*, 1993), interleukin (IL)-1β and IL-2 (Patarca *et*

al., 1993; Pollack *et al.*, 1994). Angiotensin II is also shown to induce OPN production by cardiac fibroblasts in rats with renovascular hypertension (Ashizawa *et al.*, 1996). The exact role of OPN in the regulation of inflammation is not yet clarified with both pro and anti-inflammatory reactions reported in different conditions as discussed below.

There is evidence of pro-inflammatory effects from *in-vitro* and *in-vivo* studies suggesting that OPN is involved in critical inflammatory processes including recruitment of inflammatory cells such as macrophages, T cells and astrocytes (Ellison *et al.*, 1999; Lampe *et al.*, 1991; O'Regan *et al.*, 1999) and in cytokine expression (Attur *et al.*, 2000). Additionally, OPN null mice studies support the role of OPN in inflammation. In one study, OPN null mice with obstructed kidney displayed significantly lower number of infiltrating macrophages compared to wild type counter-parts (Ophascharoensuk *et al.*, 1999). In another study in mice with chemical induced squamous cell carcinoma, the number of macrophages was lower in OPN null mice compared to wild type controls (Crawford *et al.*, 1998).

Paradoxically, OPN also exhibits anti-inflammatory actions such as inhibition of NO production by macrophages as well as reducing oxidative stress levels (Guo *et al.*, 2001; Hwang *et al.*, 1994a). OPN inhibits iNOS and NO release by macrophages *in-vitro* in a dose dependent manner (Rollo *et al.*, 1996b). In post-ischemic kidneys of OPN null mice, levels of iNOS and nitrotyrosine (an indicator of NO levels *in vivo*) were increased to a markedly higher level than in wild type post-ischemic kidneys (Noiri *et al.*, 1999). In addition, OPN has been demonstrated to be a negative feedback regulator of NOS in murine macrophages (Guo *et al.*, 2001; Scott *et al.*, 1998). In these studies, NO stimulates expression of OPN which inhibits iNOS transcription and reduces NO production. In rheumatoid arthritis and osteoarthritis, the synovial fluid of the affected joints shows increased OPN expression and OPN is reported to inhibit production of the pro-inflammatory cytokines NO and prostaglandin E_2 (Attur *et al.*, 2001; Petrow *et al.*, 2000). Moreover, OPN also acts to decrease oxidative stress levels in injured tissue (Hwang *et al.*, 1994a).

In addition, OPN may also have a role in tissue repair as it helps recruit inflammatory cells, an action that is not only important for inflammation but also for healing and tissue repair. In fact, studies in OPN null mice support a role of OPN in

tissue repair. Following incisional wounds, OPN null mice showed less matrix organisation, more residual debris and altered collagen fibrillogenesis (Liaw *et al.*, 1998a).

1.4.3 OPN in cell survival

Evidence shows that OPN may have the ability of enhancing cell survival through its anti-apoptotic actions. It inhibits apoptosis in smooth muscle, endothelial cells, epithelial cells, macrophages and pro-B cells amongst others (Weber, 2001).

There is a large body of evidence from different tissue and cell types supporting the role of OPN in cell survival. OPN was shown to protect endothelial cell lines from serum deprivation induced apoptosis (Rice *et al.*, 2006). Other studies demonstrated neuroprotective effects of recombinant OPN in *in-vitro* and *in-vivo* models of ischemia (Doyle *et al.*, 2008; Meller *et al.*, 2005). In neonatal rats subjected to hypoxia-ischemia, OPN reduced brain injury and improved neurological outcomes by inhibiting neuronal apoptosis (Chen *et al.*, 2011). Recently, OPN was shown to significantly increase survival and improve morphology of porcine retinal neurons from a transgenic mouse model of retinal degeneration (Del Río *et al.*, 2011). OPN has also been suggested to enhance macrophage survival and accumulation in the brain using *in-vitro* and *in-vivo* models of neuroAIDS, HIV infections of the CNS (Burdo *et al.*, 2007). In addition, OPN KO studies provided further evidence for the pro-survival effects of OPN. Apoptosis of interstitial and tubular cells is increased in obstructed kidneys of OPN KO mice compared to their wild type equivalents (Ophascharoensuk *et al.*, 1999). In the same study, treatment of serum-starved renal epithelial cells with anti-OPN antibodies significantly increased apoptosis. In a separate study, OPN null mice exhibited higher post-ischemic apoptosis in kidney tubular cells compared to wild type controls (Persy *et al.*, 2003).

The exact mechanism of the pro-survival effects of OPN is not yet established but there have been studies in endothelial cell lines showing that OPN inhibits apoptosis by activating NF-kB via its $\alpha_v\beta_3$ receptor (Rice *et al.*, 2006; Scatena *et al.*, 1998). Another suggested mechanism is by increasing levels of the anti-apoptotic protein Bcl-X_L within the cell and preventing dispersal of BCL-$X_{L/S}$ (Denhardt *et al.*, 1998; Khan *et al.*, 2002). Moreover, OPN was shown to increase survival of gastric

cancer cells by interacting with variant isoforms of CD44 and through activation of β_1 integrins (Lee *et al.*, 2007) and its presence constitutively within a breast cancer cell line was shown to inhibit apoptosis after chemotherapy treatment (Graessmann *et al.*, 2006). OPN showed a similar effect on dexamethasone-induced apoptosis in multiple myeloma cells which was inhibited by anti-CD44v6 antibodies (Caers *et al.*, 2006) suggesting that OPN may exert its anti-apoptotic actions through ligation with the CD44 receptor. On the other hand, the neuroprotective effects of OPN against ischemia induced cell death in primary cortical neurons was inhibited by peptide integrin inhibiors, implying the involvement of integrin receptors in the protective actions (Meller *et al.*, 2005). Both in the stroke and retinal neurodegeneration models, OPN pro-survival effects were mediated through activation of PI3K/Akt pro-survival pathway (Del Río *et al.*, 2011; Meller *et al.*, 2005).

1.5 OPN in the CNS

A comprehensive investigation of the distribution of OPN expression in the brain has not yet been undertaken, but there are various reports on its expression in specific areas or whole brain lysates. Some have reported no expression of OPN protein in whole rat brain lysates using Western blotting (Satoh *et al.*, 2008) or rat hippocampus using immunohistochemistry (Choi *et al.*, 2007). By contrast, others report evidence of OPN expression in the septal nucleus and ventral brain nuclei in the same species (Ellison *et al.*, 1998). In these laboratories, evidence of OPN expression was found in the SN of rats (Iczkiewicz *et al.*, 2004) and marmosets (Iczkiewicz *et al.*, 2006). In mice, no OPN expression could be obtained in the hippocampus using immunohistochemistry (Wirths *et al.*, 2010). However, in the human brain OPN is expressed in hippocampal pyramidal neurons (Wung *et al.*, 2007) and dopaminergic neurons of the SN (Iczkiewicz *et al.*, 2006). In contrast, Western blot analysis of whole human brain lysates showed no OPN expression in normal brains (Satoh *et al.*, 2008). This may be due to the use of a whole brain lysate rather than SN only, which may have reduced the concentration of OPN below detection levels.

OPN is up-regulated in a number of models of disorders of the CNS. It is up-regulated in response to status epilepticus in degenerating neurons and axons in the rat hippocampus (Borges *et al.*, 2008). In kainic acid induced seizures in rat, OPN

was up-regulated but localised to activated microglia (Kim *et al.*, 2002). It is also up-regulated in a mouse model of Alzheimer's disease (AD) (Wirths *et al.*, 2010) and in pyramidal neurons in brains of AD patients compared to age-matched controls (Wung *et al.*, 2007). OPN is transiently up-regulated in microglia and prolonged up-regulation is seen in astrocytes, following transient forebrain ischemia in rats (Choi *et al.*, 2007). Up-regulation of OPN in CNS disorders suggests that it is either part of the homeostatic defence in the CNS or a deleterious agent with detrimental effects contributing to the disorder. Some evidence suggests that OPN has a protective role in the CNS. For example, OPN null mice showed greater thalamic neurodegeneration post ischemic stroke compared to wild type controls (Schroeter *et al.*, 2006), suggesting that the role of OPN in ischemic injury is protective. This concept is supported by the evidence of neuroprotective and pro-survival effects of OPN in models of ischemia (Section 1.4.3). On the other hand, treatment of OPN null mice with OPN did not affect neurodegeneration or inflammation following status epilepticus (Borges *et al.*, 2008).

Additionally, evidence suggests that OPN is up-regulated in inflammatory paradigms centrally. It is up-regulated in lysosomal storage disorders associated with inflammation, like Sandhoffs- and Tay-Sachs-disease (Myerowitz *et al.*, 2002) and in rat cerebral cortex post traumatic injury where it co-localised with ED-1 positive macrophages and activated microglia (von Gertten *et al.*, 2005). In cryolesioned rats, OPN expression was also up-regulated in the cerebral cortex where it co-localised with activated microglia and macrophages in addition to some neurons and astrocytes (Shin *et al.*, 2005). Expression of OPN was increased significantly in the scrapie-infected brains compared to controls and was mainly expressed in neurons but also in reactive astrocytes and some microglia (Jin *et al.*, 2006). In addition, OPN is discussed to act as a chemoattractant for astrocytes following ischemic injury (Ellison *et al.*, 1999; Ellison *et al.*, 1998; Wang *et al.*, 1998). Further, systemic LPS injection in rats induced transient OPN expression in parenchymal astrocytes and microglia in the brain, supporting the suggested inflammatory role of the protein (Choi *et al.*, 2003).

The role of OPN in inflammation in the CNS was clarified by a number of studies. OPN null mice with spinal cord injury display lower inflammation, larger tissue damage and impaired locomotor recovery suggesting a neuroprotective role of

OPN in inflammatory responses (Hashimoto *et al.*, 2007). Also, OPN null mice exhibited higher microglial activation and inflammatory gene expression following ischemic cortical stroke compared to wild type animals (Schroeter *et al.*, 2006). In another study, following transient forebrain ischemia in rat, OPN was up-regulated in microglia and astrocytes and secreted into the extracellular space by astrocytes suggesting a role in tissue repair (Choi *et al.*, 2007). This is in line with other reports of neuroprotective effects of OPN in models of stroke (Doyle *et al.*, 2008; Meller *et al.*, 2005). On the other hand, in multiple sclerosis and its animal model experimental autoimmune encephalomyelitis, OPN is reported to be up-regulated mainly in microglia and macrophages and to increase survival and proliferation of T-cells resulting in a harmful pro-inflammatory effect (Chabas *et al.*, 2001; Hur *et al.*, 2007; Jansson *et al.*, 2002; Kim *et al.*, 2004). This difference may be due to the difference in disease models as multiple sclerosis is a T-cell mediated autoimmune disease and the former two disorders are ischemia or injury mediated neurodegeneration. The evident involvement of OPN in the regulation of inflammation in the brain suggests that it may play a role in regulating this process in PD. Nevertheless, the effect of OPN on the inflammation in PD has not been previously investigated.

1.6 Receptors with which OPN interacts

OPN exerts its actions via integrin and CD44 receptors, which are ubiquitous multifunctional and multistructural transmembrane glycoproteins.

1.6.1 Integrins

Integrin receptors are involved in the regulation of many aspects of cell behaviour such as differentiation, adhesion, migration and apoptosis (Barczyk *et al.*, 2010). They consist of α and β subunits which bind non-covalently to form the integrin receptor heterodimer (Figure 1.3). So far, 18 α and 8 β subunits have been identified and they bind to form 24 combinations of integrin receptors (Jalkanen *et al.*, 1986). OPN can mediate cell-cell and cell-matrix interactions by binding several integrins. Through the RGD domain, it binds $\alpha_v\beta_3$, $\alpha_v\beta_1$, $\alpha_v\beta_5$, $\alpha_5\beta_1$ and $\alpha_8\beta_1$ (Attur *et al.*, 2000; Bayless *et al.*, 1998; Denda *et al.*, 1998; Hu *et al.*, 1995; Liaw *et al.*, 1994; Smith *et al.*, 1996; Yue *et al.*, 1994). Whereas binding to $\alpha_4\beta_1$ and $\alpha_9\beta_1$ appears to be RGD-independent; binding is instead to the SVVYGLR sequence in OPN but only

after thrombin cleavage (Bayless *et al.*, 2001; Smith *et al.*, 1996; Yokasaki *et al.*, 2000). In contrast, Barry *et al*., report activation of $\alpha_4\beta_1$ by the full length protein (Barry *et al.*, 2000).

The interaction of OPN with integrin $\alpha_v\beta_3$ has been implicated in cell attachment and migration whilst interaction with $\alpha_v\beta_1$ and $\alpha_v\beta_5$ mediates adhesion but not migration (Giachelli *et al.*, 1995; Liaw *et al.*, 1995). Integrins α_4 and α_5 may also mediate adhesion (Nasu *et al.*, 1995) and $\alpha_9\beta_1$ may induce cellular migration (Smith *et al.*, 1998). $\alpha_v\beta_3$ is the most characterised integrin receptor of OPN and some claim that OPN has the highest specificity for $\alpha_v\beta_3$ (Caltabiano *et al.*, 1999). $\alpha_v\beta_3$ integrin receptor appears to be important for OPN-mediated NF-$_K$B induction and survival, as adding a neutralizing anti-β_3 integrin antibody blocked NF-$_K$B activity induced by OPN (Scatena *et al.*, 1998). In the same study, β_1 integrin mediated laminin but not OPN induced cell survival, this is contradictory to reports of involvement of this particular integrin in OPN-mediated cell survival (Lee *et al.*, 2007).

Pinkstaff *et al*. have mapped the expression of many integrins' mRNA expression in the rat brain (Pinkstaff *et al.*, 1999). They found no expression of integrin α_4 (Igα_4), Igα_v, Igα_8 mRNA in the SN. The mRNA for Igβ_1, Igβ_5 and Igα_5 receptors was present in the SN. Expression of Igα_9 mRNA has not been investigated. The group also report that they could not detect an mRNA signal for β_3 integrin, although they describe in the same paper unpublished Western blotting data suggesting presence of this integrin in the brain (Pinkstaff *et al.*, 1999). A more detailed investigation of the expression of these receptors in SN and their sub-cellular localisation has not been undertaken.

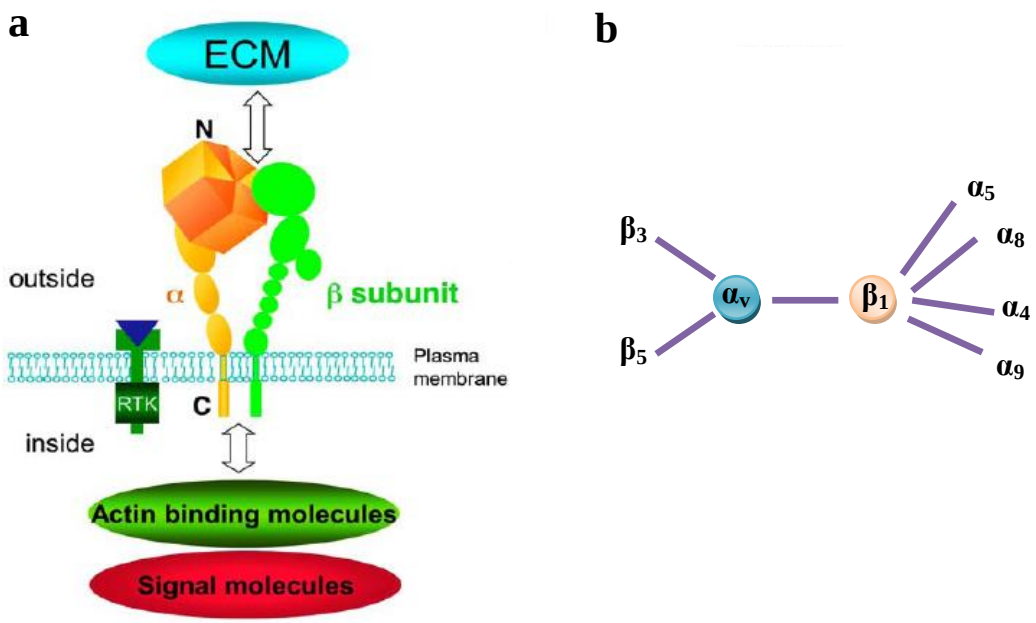

Figure 1.3 Integrin receptors.

(a) Protein structure of the integrin receptor. Integrin receptors are heterodimmers of non-covalently bound α and β subunits. They mediate signals from outside of cell to the inside (outside-in) and from inside the cell to the outside (inside-out). Adapted from (Gu *et al.*, 2004).

(b) Integrin dimmers that bind to OPN. There are **7** integrin receptors known to bind OPN so far. These are combinations of either α_v or β_1 integrins and α_4, α_8,α_5 ,α_9, β_3 or β_5.

1.6.2 CD44

CD44, the main receptor of hyaluronic acid, has several isoforms serving diverse functions. The standard isoform CD44s is the most abundant and there exist many splice variants termed CD44v. These are the result of combination of exons 6-14 of the gene, which can be alternatively spliced and inserted into the extracellular domain (Figure 1.4) (Naor *et al.*, 1997). CD44 is expressed on many cell types including epithelial cells, keratinocytes, fibroblasts, leukocytes, macrophages and endothelial cells (Fox *et al.*, 1993; Gunthert *et al.*, 1991). In fact, CD44 was shown to be expressed on dopaminergic neurons of the SN (Fuxe *et al.*, 1996). CD44 is involved in mediating cell-cell and cell-matrix interactions thereby facilitating cell adhesion (Aruffo *et al.*, 1990), migration (Peck *et al.*, 1996; Trochon *et al.*, 1996), lymphocyte homing (Jalkanen *et al.*, 1986; Streeter *et al.*, 1988) and activation (Conrad *et al.*, 1992; Huet *et al.*, 1989), hyaluronate degradation (Underhill, 1992),

angiogenesis (Trochon *et al.*, 1996) and release of cytokines (Webb *et al.*, 1990). In addition, OPN is involved in CD44 mediated chemotaxis (Weber *et al.*, 1996b). Further, CD44 expression in tumour cells is associated with tumour progression and invasiveness (Gunthert *et al.*, 1991; Sy *et al.*, 1991) probably through its ability to mediate cell migration. In addition, CD44 is upregulated in proliferating cells including cells involved in tissue repair, morphogenesis, organogenesis and angiogenesis (Trochon *et al.*, 1996; Underhill, 1992; Yu *et al.*, 1997).

Some evidence suggests that OPN binds variant isoforms of CD44 but not the standard CD44s. Weber *et al.* (1996) showed that a splice variant of CD44 present in fibroblasts namely (CD44v7-v10) facilitates adhesion and migration to OPN (Weber *et al.*, 1996a). In addition, not only had v6 or v7 to be present in CD44 in order to bind OPN but also RGD independent interaction with β_1 integrin is required (Katagiri *et al.*, 1999). OPN binding to *both* CD44v6 and RGD-binding integrins was shown to be essential for the proliferation and migration of 5TMM cells (Caers *et al.*, 2006) and binding to CD44 is essential for migration of macrophages to injury site (Marcondes *et al.*, 2008). Furthermore, OPN inhibition of apoptosis in gastric cell lines; this action was dependant on the expression of CDDv6 and v7 isoforms and was inhibited by anti-integrin β_1 antibody and RGD peptides. In contrast, OPN has been shown to bind CD44 receptor variants (v6 and v7) by an RGD-independent mechanism (Denhardt *et al.*, 2001; Weber *et al.*, 1996a) inducing macrophage accumulation and engagement of the β_3 integrin (Weber *et al.*, 1996a).

Integrin and CD44 receptors are known to mediate OPN effects in the periphery and some central systems but it is not known whether actions of OPN in the SN, if any, are mediated through these receptors.

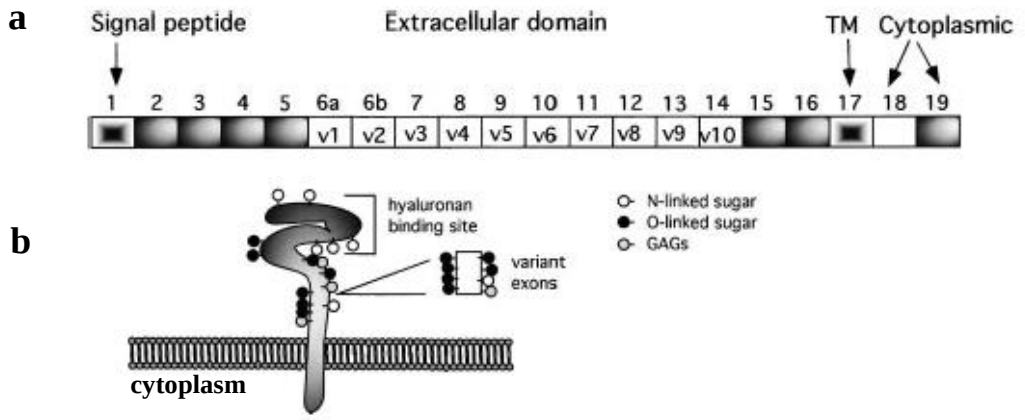

Figure 1.4 Structure of CD44 gene and protein.

(a) Schematic gene structure. v1-v10 boxes represent the 10 variant exons. Exon 18 is spliced out to allow transmembrane (TM) domain to be followed by cytoplasmic domain. (b) Protein structure. CD44 has a highly conserved 21 amino acid transmembrane domain and 72 amino acid cytoplasmic domain. The extracellular domain comprises of: a highly conserved amino terminal domain 80% homology, a relatively conserved (35-45% homology) membrane proximal domain containing several glycosilation sites and between these two regions is the region where the variant exons can be inserted producing variant isoforms of CD44 (CD44v). Adapted from (Isacke *et al.*, 2002). GAGs: glycosaminoglycans.

1.7 OPN in PD

OPN plays an important role in the inflammatory process. It is up-regulated in the early stages of inflammation and has pro-inflammatory and anti-inflammatory actions. Inflammation is an important element of the underlying pathogenesis of PD, and thus OPN has a potential involvement in the regulation of inflammatory pathology of PD. Importantly, OPN effects may be age related, age being an established factor that increases risk of developing PD. The anti-inflammatory actions of OPN, namely inhibiting NO release by macrophages, inhibiting NOS, and decreasing oxidative stress levels appear to decline with age (Guo *et al.*, 2001; Rollo *et al.*, 1996a; Scott *et al.*, 1998) suggesting that replacing OPN in age related disorders such as PD may be neuroprotective.

Recently, OPN was found to be present in the rat basal ganglia with highest levels in the SN, the main site of pathology in PD (Iczkiewicz *et al.*, 2004). Both OPN protein and mRNA levels are up-regulated after supra-nigral LPS injection

(Iczkiewicz *et al.*, 2005) where it co-localised with microglia, suggesting that OPN may be involved in regulating inflammation mediated neurodegeneration. Conversely, OPN expression was reduced following MPTP challenge in marmosets and was not co-localised with glia (Iczkiewicz *et al.*, 2006). These results may not be contradictory but instead reflecting different phases of OPN regulation. This is because in the first study, OPN expression was investigated within five days of LPS lesioning while in the second study it was examined 18 months following MPTP treatment. OPN was also found to be present in dopaminergic neurones in normal human brain (Iczkiewicz *et al.*, 2006). Importantly, in PD patients, protein expression was decreased in remaining dopaminergic neurones and was present in activated microglia (Iczkiewicz *et al.*, 2006). In a separate study, patients with PD had increased OPN expression in the SN and higher OPN levels in serum and CSF compared to controls and higher levels were associated with more severe symptoms (Maetzler *et al.*, 2007). In the same study, OPN deficient mice showed reduced degeneration of dopaminergic neurons following MPTP challenge compared to wild type animals (Maetzler *et al.*, 2007) suggesting that OPN may have a detrimental role in PD. These contradictory findings are not easily explained but both seem to point to a role of OPN in PD. Moreover, a recent study showed that a single-nucleotide polymorphism of the OPN gene may be linked to susceptibility to LB diseases including PD (Maetzler *et al.*, 2009).

OPN has been shown to prevent apoptosis in different cell types, to be involved in cell regeneration and tissue repair and to modulate inflammation. This supports the hypothesis that OPN may have neuroprotective properties in PD and this will be investigated in this thesis.

1.8 Hypothesis

There is a large body of evidence suggesting the involvement of OPN in pro-survival mechanisms. In addition, OPN seems to play a protective role in diverse tissues. In kidney, OPN appears to protect against renal stone formation and in bone, it plays an important role in remodelling. In the heart, this protein is shown to prevent tissue calcification and is suggested to promote tissue repair. In addition, OPN promotes survival of tumour cells and has an important role in regulating inflammation both peripherally and centrally.

Based on evidence of the involvement of OPN in the regulation of inflammation and its pro-survival effects it was hypothesised that OPN protects dopaminergic neurons against toxin insults through anti-inflammatory effects, mediated via binding to $Ig\alpha_v$, $Ig\beta_3$, $Ig\beta_1$ or CD44 receptors.

1.9 Aims

- To determine whether endogenous expression of OPN is intrinsically neuroprotective.
- To determine whether OPN exogenous treatment is neuroprotective.
- To investigate the role of glial cells in the neuroprotective effects of OPN.
- To investigate the role of $Ig\alpha_v$, $Ig\beta_3$, $Ig\beta_1$ and CD44 receptors in the neuroprotective effects of OPN.

Chapter 2 Materials and methods

2.1 Introduction

The aims of this thesis were to investigate the neuroprotective effects of OPN in cell lines, to study whether its effects are related to the endogenous expression of the protein and then to investigate the effects of OPN in more complex paradigms; the VM primary culture and rat brains. Initial studies were carried out in each experimental model to characterise it for the expression of OPN and receptors involved with its cell survival actions namely Igα_v, Igβ_3, Igβ_1 and CD44. Then, neuroprotection studies were carried out in the three characterised models to test the ability of OPN pre-treatment to protect dopaminergic cells from toxin induced cell death. The methods used in these studies are outlined in detail below.

2.2 Cell lines

Five cell lines derived from different cell types from rat, mouse and human were used: Ntera-2, a human neurone committed teratocarcinoma cell line (Angulo *et al.,* 1995); SK-NMC, a human neuroblastoma cell line with cholinergic characteristics (Biedler *et al.,* 1973; Biedler *et al.,* 1978); PC-12, rat pheochromocytoma cell line that differentiates into sympathetic-like neurons in response to nerve growth factor (NGF) and extracellular matrix molecules (Greene *et al.,* 1987); N1E-115 cell line, mouse adrenergic neuroblastoma clone with highest levels of TH (Amano *et al.*, 1972). These cell lines were obtained from the American Type Culture Collection. SH-SY5Y, a human catecholaminergic neuroblastoma cell line derived from SK-N-SH cell line established from a bone marrow biopsy (Biedler *et al.,* 1973) was obtained from The European collection of cell cultures.

2.2.1 Maintenance of cells

SH-SY5Y, SK-NMC, N1E-115 and Ntera-2 cells were maintained in tissue culture flasks (NUNC, Thermo scientific, UK) in Dulbecco's Modified Eagle's Mediu (DMEM-Glutamax), supplemented with foetal bovine serum (FBS; 10%), penicillin (5mg/ml), streptomycin (5mg/ml) and Neomycin (10mg/ml) (PSN, 1%) in a humidified 5% CO_2 chamber (Sanyo, UK) at 37˚C. Medium was changed every two to three days and cells were detached using trypsin (0.25%) at about 80% confluence and seeded into new flasks in full growth medium. PC-12 cells were maintained in

the same manner but the medium used was Kaighn's nutrient mixture supplemented with horse serum (15%), FBS (2.5%) and PSN (1%).

2.2.2 Cell counting

An aliquot of the cell suspension (50µL) was added to Trypan blue solution (50µL; 0.5%) and live cells which do not take up the dye were counted using a haemocytometer (Neubauer, Germany) under TMS inverted microscope (Nikon, UK) at x0.25 magnification. Cells were counted in four corner squares, each square has a surface area of $1mm^2$ and represents a total volume of $10^{-4}cm^3$. Since 1cm3=1ml, the following equation was used to estimate the number of cells per 1ml:
Cells per ml = The average count of cells per square x the dilution factor x 10^4

2.2.3 Cryopreservation of cells

When cell cultures reached 80% confluence, they were detached with trypsin (0.25%) and centrifuged at 950g for 5min in a CR3i centrifuge (Jouan, DJB Labcare, UK). Cells were re-suspended at 1.5-2x10^6cell/ml in the cryoprotectant solution (full growth culture medium (Section 2.2.1) with FBS (40%) and dimethyl sulfoxide (20%) and placed in cryo-vials (2ml; Corning Inc., UK) in a Mr Frosty freezing container (Nalgene, USA) at -70˚C for 24h then transferred to liquid nitrogen (-200˚C).

2.2.4 Thawing cells from cryoprotection

Cryo-vials were removed from liquid nitrogen and directly placed in a 37˚C water bath for 1-2min. Once thawed, the cryoprotectant solution containing the cells was slowly added to pre-warmed full growth medium (10ml; details in Section 2.2.1) and centrifuged at 950g for 5min (CR3i centrifuge, Jouan, DJB Labcare, UK). After discarding the supernatant, cells were re-suspended in full growth medium and grown as described in Section 2.2.1.

2.2.5 Preparing cells for immunostaining

2.2.5.a Coating of coverslips

Glass coverslips (13 mm diameter; Scientific Laboratories supplies, UK) were washed three times in ethanol (70%) then rinsed three times with distilled water. Coverslips were sterilised in an autoclave at 121˚C for 30min and air-dried, then

immersed in poly-D-lysine solution (0.01% in sterile distilled water) for 30min. Coverslips were rinsed twice with sterile distilled water to wash off the unbound coating material before use.

2.2.5.b Growing cell lines for immunostaining

For immunofluorescence, cells (1.5-2x10^5cell/well) were grown on poly-D-lysine coated glass cover slips in twenty four well plates (Nunc, Thermo scientific, Germany) in culture medium (0.5ml/well; Section 2.2.1). At about 60% confluence, culture medium was discarded and cells washed twice with cold Dulbecco's phosphate buffered saline (DPBS, pH 7.4; 0.5ml/well). The cells were then fixed in cold paraformaldehyde (PFA, 0.4% in 0.1M Phosphate buffered saline (PBS)) for 20min.

2.2.6 Fluorescence immunocytochemistry in cell lines

This technique was carried out in 24 well plates (0.5ml/well). After fixation (Section 2.2.5.b), cells were washed twice with PBS (0.1M) and incubated with 20% blocking solution containing goat serum (20%), Triton-X 100 (0.05%)/0.1M PBS for one hour. The cells were then incubated with primary antibodies (details in Table 2.1) diluted in 1% blocking solution (goat serum, 1%; Triton-X 100, 0.05%/0.1M PBS) overnight at 4˚C followed by three 5min washes with PBS (0.1M). Next, cells were incubated with the appropriate secondary antibodies (Table 2.2) conjugated with Texas red or Fluorescein for one hour in a darkened environment. After three 30min washes with PBS (0.1M), coverslips were mounted, in the dark, onto polysine slides (Menzel-glaser, Germany) using vectashield hardset mounting medium with 4',6-diamidino-2-phenylindole (DAPI) a fluorescent stain that binds strongly to DNA. A Zeiss Axioskop 40 microscope (Germany) was used to examine the cells. Specificity of antibodies was tested by omitting the primary antibody. Figure 2.1 shows typical TH and OPN staining in cell lines.

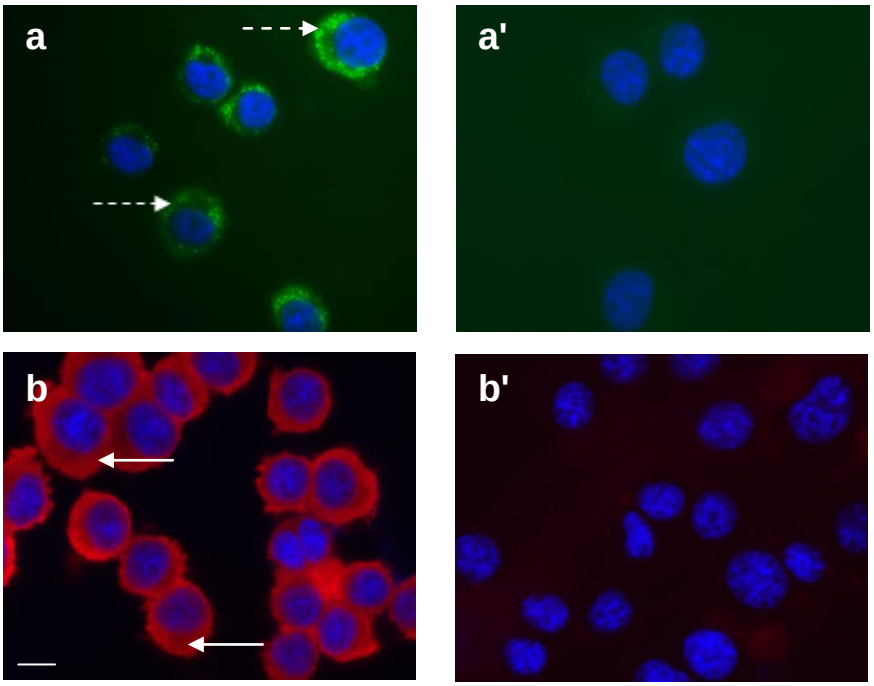

Figure 2.1 Typical TH and OPN Immunofluorescence in PC-12 cells.

PC-12 cells show positive immunoreactivity to (a) OPN (green; - - ►) in the cytoplasm and to (b) TH (red; ⟶) in the cytoplasm. DAPI counter stain is shown in blue. (a') Primary antibody omitted, fluorescein conjugated anti-mouse secondary antibody used. (b') Primary antibody omitted, Alexa Fluor conjugated anti-rabbit secondary antibody used. Magnification x100, Scale bar = 20µm and is representative of all images.

Antibody	Supplier	Species	Dilution (IF)	Antigen
Anti-TH	Sigma-Aldrich, UK	mouse	1:200	TH
Anti-OPN	Hybridoma bank, Canada	mouse	1:200	OPN
Anti-Igα_v	Santa cruz biotechnology, Germany	rabbit	1:100	Integrin α_v receptor
Anti-Igβ_3	Santa cruz biotechnology, Germany	rabbit	1:50	Integrin β_3 receptor
Anti-Igβ_1	Santa cruz biotechnology, Germany	rabbit	1:50	Integrin β_1 receptor
Anti-CD44, fluorescin conjugated	Abcam, UK	rat	1:100	CD44 receptor

Table 2.1 Primary antibodies used in cell lines with their respective details and dilutions.

Immunofluorescence (IF).

Antibody	Supplier	Species	Dilution (IF)
Fluorescin conjugated anti-mouse IgG	Jackson immunolabs, UK	goat	1:200
Texas Red conjugated anti-rabbit IgG	Vector Laboratories, UK	goat	1:100

Table 2.2 Secondary antibodies used in cell lines and their respective dilutions and details.

Immunofluorescence (IF).

2.2.7 Western blotting

2.2.7.a Preparing cells for Western blotting

Cells were grown in full growth medium (35ml; Section 2.2.1) in 75 cm^2 flasks until they reached approximately 60-80% confluence (Section 2.2.1). Growth medium was removed and cells washed three times with DPBS (10ml). Lysis buffer (600µl; Tris-Hcl buffer 50mM, sodium dodecyl sulphate (SDS; 1%)) was then added to the flask and cells were collected by scraping using a sterile scraper (BD Falcon, UK). The cell suspension was triturated ten times through a Needle (25G, 1.5'') using a 1 ml syringe in order to ensure complete lysis of cells. The cell lysate (600µl) in an eppendorf tube (alpha laboratories, UK) was then heated at 94˚C in a standard heating block (VWR, UK) for 10min to linearise the proteins and then centrifuged at 75590g for 10min in a micro-centrifuge (Galaxy 14D, VWR, UK) and the supernatant was retained and stored at -70˚C until use. Protein concentration was determined using the protein A280 Nano drop ND-1000 spectrophotometer assay (Labtech international, UK).

2.2.7.b Nano drop protein A280 assay

Samples (2µl) were loaded on the measurement pedestal of the Nano drop spectrophotometer and absorbance measured at 280nm three times for each sample. Lysis buffer (2µl) was loaded on the measurement pedestal and measured as 'blank measurement'. Protein concentration in (mg/ml) is calculated by the program using absorbance by bovine serum albumin (BSA) solution as a reference. Figure 2.2 shows a typical absorbance spectrum. Accuracy and linearity of protein concentration measurement was checked by diluting BSA protein standard a series of dilutions (0.4-100mg/ml) and comparing the actual concentrations with measured concentrations (Figure 2.3). The concentrations obtained for cell lines (10-25mg/ml) were within the very accurate range of the Nanodrop assay (Figure 2.3).

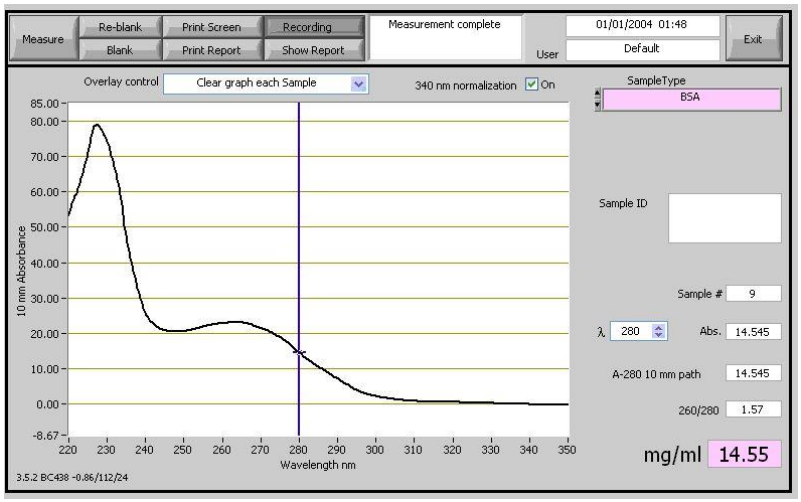

Figure 2.2 Typical absorbance spectrum using the Nanodrop spectrophotometer Protein A280 program.

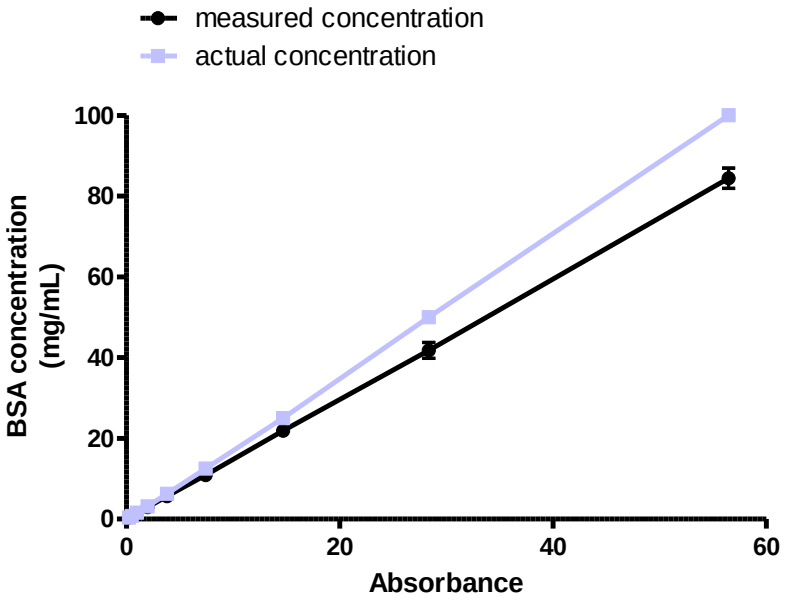

Figure 2.3 BSA actual concentration vs. Nanodrop-measured concentration.

BSA protein standard solutions of known concentrations (0.4-100mg/ml) were measured using the Nanodrop assay in order to compare actual concentration with measured concentration.

2.2.7.c Preparation of positive control

Male Wistar rats were terminally anaesthetised with sodium pentobarbitone (100mg/kg, i.p.), the kidneys removed then snap frozen immediately at -45˚C in isopentane. The tissue was homogenised using a motor pellet pestle (Sigma) in Lysis buffer (Tris base, 50mM; TritonX-100, 1%; NaCl, 150mM; EDTA, 5mM; PMSF, 2mM and protease inhibitor cocktail set III, 5%) at a concentration of 1mg/4µl then centrifuged at 10.000g at 4˚C for 30min (Heraeus microcentrifuge, DJB labcare, UK). The supernatant was decanted and stored at-70˚C until use.

2.2.7.d Gel electrophoresis

SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis) was used to separate the proteins. The gel comprised of a stacking gel (acrylamide, 2%; N,N, Methylene-bis-acrylamide, 0.08%; SDS, 0.1%; TEMED, 0.1%; ammonium picosulphate, 0.05% and Tris, 125mM, pH 6.8) and a resolving gel (acrylamide, 12.5% ; N,N, Methylene-bis-acrylamide, 0.42% ; SDS, 0.04%; TEMED, 0.77%; ammonium picosulphate, 0.1% and Tris, 375mM, pH 8.8).

Aliquots of cell lysates containing 100µg protein or rat kidney homogenate (10µg protein) were added to loading buffer (Tris, 5mM, pH 6.8; SDS, 2.5%; glycerol, 50%; bromophenol blue, 0.1% and β-mercaptoethanol, 10%) at a ratio of 1:5 (loading buffer : sample) and were loaded on 12.5 % SDS PAGE gels. The gel was placed in a gel caster system (Biorad, UK) and electrophoresis was carried out for one hour at 120V in running buffer (SDS, 0.1%; glycine, 384mM and Tris, 49.5mM) using a GIBCO BRL power supply (Life technologies, UK).

2.2.7.e Protein transfer

A polyvinylidene difluoride membrane (PVDF, Biorad, UK) was pre-wet in methanol (100%), rinsed in water then blot buffer (Tris-base, 0.582%; SDS, 0.04%; Glycine, 0.294%; methanol, 20%) for 1 min and then sandwiched together with the gel in between two layers of four filter papers which were immersed in Blot buffer for 1min before use. Proteins were transferred from the gel into the PVDF membrane using an electroblot system (200/2.0 Biorad, UK) for 30min at 19V, 200mA.

2.2.7.f Immunoblotting

Membranes were incubated in 5% blocking solution (5% non fat dry powder milk in TBS-T: Tris buffer, 50mM pH 7.5; NaCl, 150 mM; Tween 20, 0.2%) overnight at 4˚C on a shaking platform to block non-specific binding, then incubated with primary antibody (mouse anti-OPN, Hybridoma Bank) at a dilution of 1:3000 in 2.5% blocking solution (2.5% non fat dry powder milk in TBS-T) for 3hr at room temperature. After three 10min washes in TBS-T, membranes were incubated for 1hr at room temperature with horse radish peroxidase conjugated secondary antibody (vector goat anti-mouse antibody 1:2000 in 2.5% blocking solution). The membranes were then washed a further 3 times in TBS-T and incubated with ECL plus Western blotting detection kit solution (Amersham Biosciences, UK) to visualise the bands. Hyperfilms (Kodak®, UK) were apposed to the membranes for varying times (1s-5min) and developed in a Compact X4 film automatic developing machine (Xograph, healthcare Ltd, UK). Typical results obtained for OPN are shown in Figure 2.4.

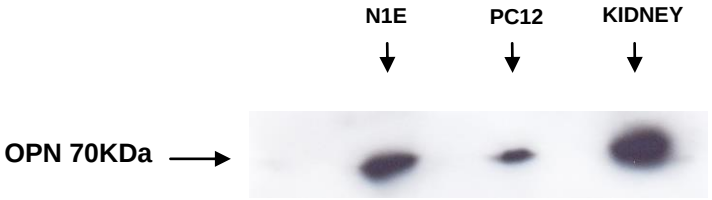

Figure 2.4 Typical Western blot showing the expression of OPN in N1E-115 and PC12 cell lines.

Cell lysates of N1E-115 and PC12 were tested for OPN expression using the Western blotting technique in the presence of positive controls (rat kidney). Both N1E-115 and PC12 cells showed a positive band for OPN.

2.2.8 Estimation of cell death

2.2.8.a LDH Assay

Cell death was determined by measuring lactate dehydrogenase (LDH) leakage from cells using the membrane integrity assay kit (Promega, UK) as this enzyme is only released from dead cells. Lysis buffer provided with the kit (2µl) was added to

3 wells of cells (100µl) in 96 well plates (NUNC, Thermo scientific, Germany). This buffer causes lysis of all cells and release of all LDH content hence it represents 100% LDH leakage. The cells were equilibrated to room temperature for 20min then assay buffer mix (100µl) was added to each well. The plates were shaken gently for 11 seconds then incubated at room temperature for 10min. Finally, stop solution (50µl) was added to each well in order to stop the reaction. Plates were read in a Spectramax Gemini XS (Molecular devices, UK), excitation 560nm emission 590nm. Background reading was taken from 3 wells containing medium only. LDH leakage (% of control) was calculated relative to the cell lysis buffer which kills all cells and represents 100% LDH release, using the following formula.

$$\% \text{ LDH leakage} = \frac{\text{Reading from well - background}}{\text{Reading from lysis buffer treated well - background}} \text{X100}$$

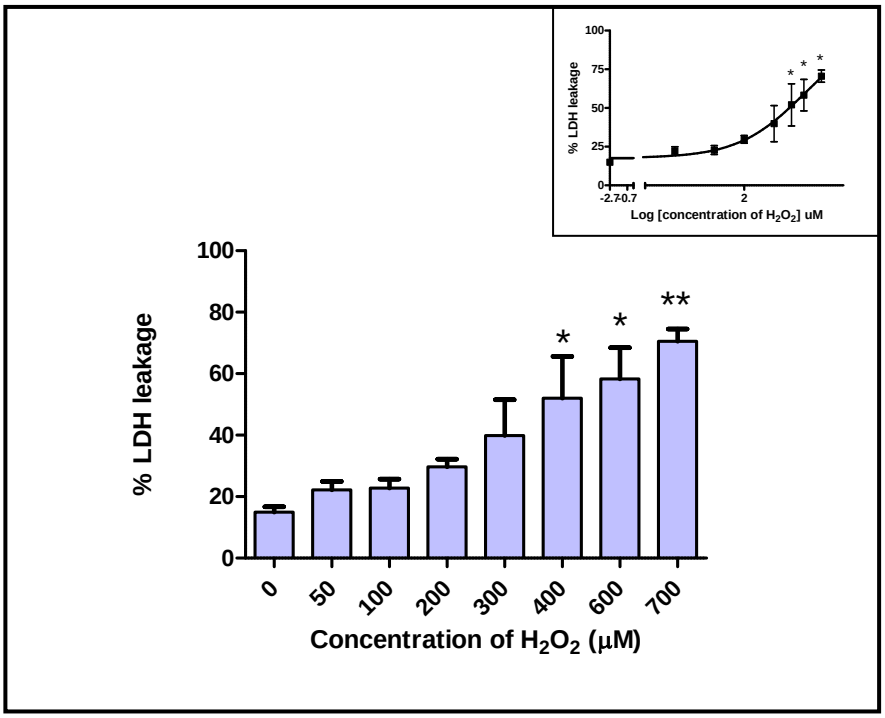

Figure 2.5 Typical results obtained with LDH assay in SH-SY5Y cells following treatment with different concentrations of H_2O_2.

SH-SY5Y cells were treated with H_2O_2 (50-800uM), then LDH assay was performed 24 hours later to assess cell death. Maximal LDH leakage from lysis buffer treated cells is 100%. Data are expressed as mean ± SEM (n=3). *P <0.05, **P <0.01, ***P<0.001 (one-way analysis of variance followed by Newman Keuls test).

2.2.8.b ATP assay

Cell death after toxin treatment was determined by measuring ATP release from cells using Cell titer glo assay kit (Promega, UK). Following equilibration of cells to room temperature for 30min, assay buffer mix (100 µl) was added to each well (100µl). The plates were shaken vigorously for 2min on a rotor shaker (VX7, Janke&Kunnel, Germany) to induce cell lysis and release of ATP from cells. Plates were incubated at room temperature for 10min to stabilise signal then read in Spectramax Gemini XS luminometer (Molecular devices, UK). ATP release (% of untreated control) was calculated using the following formula.

$$\% \text{ ATP release} = \frac{\text{Reading from well}}{\text{Reading from control untreated cells}} \text{ X100}$$

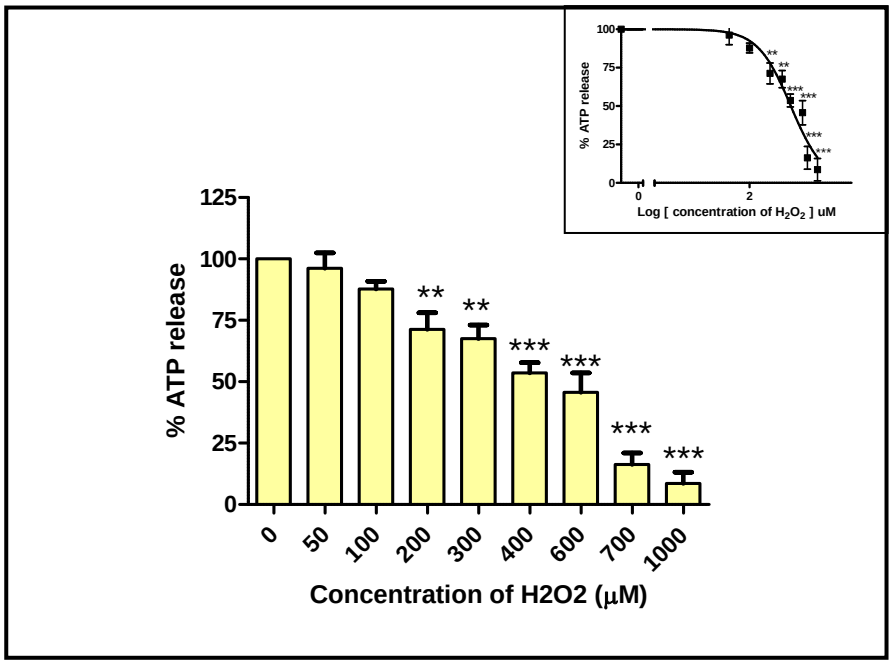

Figure 2.6 Typical results obtained with ATP content assay in SH-SY5Y cells following treatment with H_2O_2.

Cells were treated with H_2O_2 (50-800µM), then ATP content assay was performed 24 hours later to assess cell viability. Control untreated cells were taken as 100% ATP release. Data are expressed as mean ± SEM (n=3). **P <0.01, ***P<0.001 (one-way analysis of variance followed by Newman Keuls test).

2.2.8.c Data Analysis

All experiments were performed in triplicates on three to four separate occasions. Data were plotted as logarithmic values and a sigmoidal concentration-response curve was established by employing non-linear regression (Graph Pad Prism 5, USA). The software also determined the half maximal effective concentration (EC_{50}).

2.3 Primary ventral mesencephalic culture

The ventral mesencephalic (VM) region of the foetal rat brain develops in the adult rat into the SN & VTA. Unlike transformed neuronal cell lines, it contains precursors for all cell types present in the mature SN region including dopaminergic and Gamma-aminobutyric acid (GABA)-ergic neurons in addition to glial cells which differentiate when cultured *in-vitro*.

2.3.1 Dissection of the ventral mesencephalon

Time mated pregnant E14 Wistar rats (Harlan, UK) were kept under standard conditions (21˚C, 50% humidity, and 12hr light/dark cycle with free access to pelleted diet). All procedures comply with UK Animal (Scientific Procedures Act) 1986 and associated guidelines.

Animals were culled by exposure to CO_2 and E14 foetuses collected in DPBS on ice. Subsequently, foetuses were dissected under a dissection microscope (x10 magnification; Vickers Instruments, UK) by making a cut from the mouth to the VM flexure. This was followed by two sagittal cuts to separate out the mesencephalic region, one anterior and one posterior to the peak of the flexure. The ventral part of the mesencephalic segment was obtained by cutting caudally down each side of the ventricle (Figure 2.7). Finally, meninges were carefully removed and the ventral mesencephalon placed in fresh DPBS.

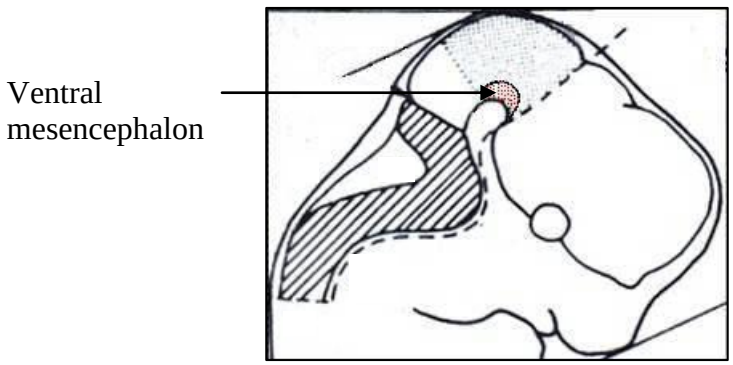

Figure 2.7 Diagram of the E14 foetal VM region.

2.3.2 Preparation of primary VM cultures

The dissected ventral mesencephalons were washed three times with DPBS in a sterile falcon tube (15ml) then incubated with trypsin (0.25%) at 37˚C for 2min. The tissue was allowed to settle and supernatant removed. Full growth medium (10ml; DMEM-GLUTAMAX, 10% FBS, 1% PSN) was then added to the tissue and centrifuged at 1369g for 1.5min (Jouan CR3i centrifuge). The supernatant was discarded and tissue pellet re-suspended in fresh medium with a micropipette followed by trituration through a fire-polished glass Pasteur pipette (pore diameter ~ 0.5mm) ten times to ensure full dispersal of cells. Cells were counted using trypan blue in the same way as cell lines (Section 2.2.2), and were plated at a density of $2x10^5$cell/well on poly-D-lysine coated coverslips in 24 well plates (full growth medium;0.5ml/well) and grown in a cell culture incubator (Sanyo, 37˚C, 5%CO_2, 100% humidity) until use.

2.3.3 Peroxidase immunocytochemistry in VM cultures

This technique was carried out in 24 well plates (0.5ml/well). At day in-vitro (DIV) 3, 4 or 5, medium was removed and cells washed with cold DPBS then fixed with cold PFA (4%/0.1M PBS) for 15min followed by two washes with PBS (0.1M). Cells were incubated in H_2O_2 (0.03%/ 0.1M PBS) for 30min to block endogenous peroxidises. After three 5min washes with 0.1M PBS (0.1M), cells were incubated with 20% blocking solution (20% goat serum, 0.05% Triton-X 100/0.1M PBS) for 1hr at room temperature. Following that, cells were incubated with primary antibodies (Table 2.3) in 1% blocking solution (1% goat serum/0.1M PBS) overnight

at room temperature. After three 5min washes with PBS (0.1M), cells were incubated with appropriate biotinylated secondary antibodies (Table 2.4) diluted in 0.05% Triton-X/0.1M PBS for one hour. After a further three 5min washes in PBS (0.1M), cells were incubated with avidin: biotinylated enzyme complex (vectastatin ABC Kit) 1:200 in 0.1M PBS for one hour. Following three 5min washes in Tris-HCL (0.05M, pH 7.4), cells were incubated with 3.3'-diaminobenzidene (DAB; 0.05% in 0.05M Tris-HCL) before addition of H_2O_2 (0.01% final concentration) to each well and colour was allowed to develop for 3min. The reaction was stopped by removal of DAB and addition of Tris-HCL (0.05M). Coverslips were then immersed in Nissl stain: Cresyl violet (0.1%) for 3min to counter stain the cell nuclei. Finally, cells on coverslips were sequentially dehydrated by dipping five times into 70%, 95% and 100% ethanol before defatting in histoclear for 5min and mounting on coverslips using DPX mounting medium. A Zeiss Axioskop 40 microscope was used to examine the cells. Specificity of antibodies was tested by omitting the primary antibody. Typical TH stained primary VM culture cells are shown in Figure 2.8.

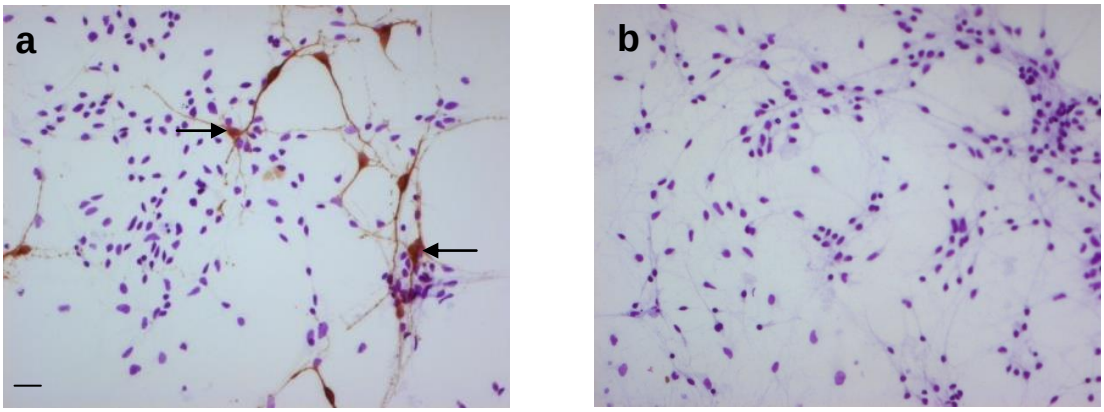

Figure 2.8 Typical TH staining in the primary VM culture.

Primary VM cultures were fixed at DIV 5 and stained by immunoperoxidase for TH (a) and counterstained with Nissl stain. Arrows show counterstained TH positive cells. (b) primary antibody omitted, biotynilated anti-rabbit secondary antibody used. Magnification x20. Scale bar =50μm and is representative of both images.

2.3.4 Cell counting

Counting of immunopositive cells was carried out on three to four coverslips per culture and each experiment was performed in three to four separate cultures. Mounted stained cultures were analysed under Zeiss Axioskop 40 microscope at magnification x10 in a blinded manner. The number of positively stained cells was counted in ten randomly selected areas on each coverslip using an eye piece grid (total area = $10mm^2$). For characterisation of VM culture for TH and Glutamate decarboxylase (GAD), the percentage of positive cells per coverslip was found by counting the number of DAB stained cells in five randomly selected areas (total area = $5mm^2$) on each coverslip expressed as percentage of the total number of cells which was identified by counting the number of cresyl violet stained cells in the same area.

2.3.5 Double labelling immunofluorescence in VM cultures

This technique was carried out in 24 well plates (0.5ml/well). After fixation, cells were washed twice with PBS (0.1M) and incubated with 20% blocking solution (20% goat serum, 0.05% Triton-X 100 in 0.1M PBS) for one hour. The cells were then incubated with two primary antibodies (Table 2.3) for detecting the two desired antigens, diluted in a 1% blocking solution (1% goat serum, 0.05% Triton-X 100 in 0.1M PBS) overnight at 4˚C. On the next day, after three 5min washes with 0.1M PBS, cells were incubated with the relevant secondary antibodies (Table 2.4) conjugated with Alexa Fluor 594 or Fluorescin for 2hr in a darkened environment. After three 30min washes with PBS (0.1M), coverslips were mounted, in the dark, onto polysine slides using vectashield hardset mounting medium with DAPI. A Zeiss Axioskop 40 microscope was used to examine the cells. Specificity of antibodies was tested by omitting the primary antibody. Figure 2.9 shows an example of double immunofluorescence staining in VM culture cells with TH and $Ig\beta_3$ receptor.

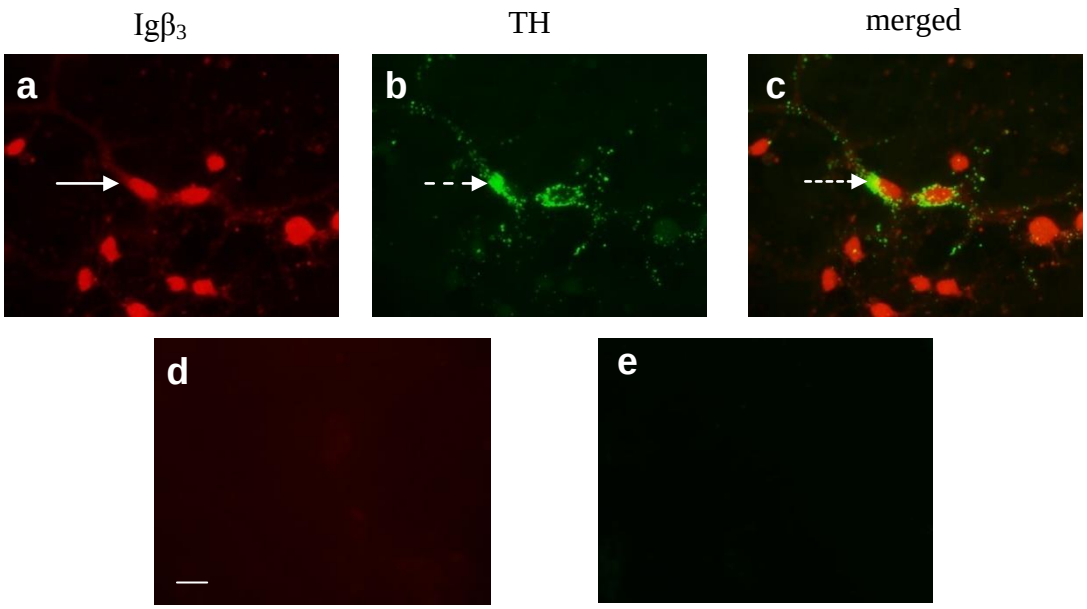

Figure 2.9 Typical double immunofluorescence staining of TH and Igβ$_3$ receptor in primary VM cells.

(a-c) Igβ$_3$ positive cells were stained and visualised in red (Alexafluor 594, ⟶) and TH positive cells were visualised in green (fluorescein, - - →). The merged picture (c) shows co-localisation between TH and Igβ$_3$ (----→) indicating that dopaminergic cells of the primary VM culture express Igβ$_3$ receptor. (d-e) Primary antibody omitted. (a) Alexafluor 594 anti-rabbit secondary antibody and (b) fluorescein conjugated anti-mouse antibody. Magnification x40, scale bar = 60µm.

Antibody	Supplier	Species	Dilution (IP)	Dilution (IF)	Antigen
Anti-TH	Pelfreeze, USA	rabbit	1:500	1:200	TH
Anti-TH	Sigma-Aldrich, UK	mouse	1:500	1:200	TH
Anti-GFAP	Calbiochem, UK	mouse	1:500	1:200	Glial fibrillary acidic protein
Anti-OX-42	AbD Serotec, UK	mouse	1:200	1:100	CR3 receptor on microglia, macrophages
Anti-OX-6	AbD Serotec, UK	mouse	1:200	1:100	MHC class II on activated microglia
Anti-ED-1	AbD Serotec, UK	mouse	1:500	1:200	single chain glycoprotein on macrophages
Anti-$GAD_{65/67}$	Sigma-Aldrich, UK	rabbit	1:1000	-	GAD
Anti-OPN	Hybridoma bank, Canada	mouse	1:500	1:200	OPN
Anti-Igα_v	Santa cruz biotechnology, Germany	rabbit	-	1:100	Igα_v receptor
Anti-Igβ_3	Santa cruz biotechnology, Germany	rabbit	-	1:50	Igβ_3 receptor
Anti-Igβ_1	Santa cruz biotechnology, Germany	rabbit	-	1:50	Igβ_1 receptor
Anti-CD44	Abcam, UK	rabbit	-	1:100	CD44 receptor

Table 2.3 Primary antibodies used in VM cultures with their respective details and dilutions.

Immunoperoxidase (IP), immunofluorescence (IF).

Antibody	Supplier	Species	Technique	Dilution
Biotynilated anti-rabbit IgG	Vector Laboratories, UK	goat	IP	1:200
Biotin conjugated anti-mouse IgG	Jackson Immunolabs, UK	goat	IP	1:200
Biotynilated anti-mouse IgG	Vector Laboratories, UK	goat	IP	1:200
Fluorescin conjugated anti-mouse IgG	Jackson immunolabs, UK	goat	IF	1:200
Alexa Fluor 488 anti-mouse IgG	Invitrogen, UK	donkey	IF	1:200
Alexafluor 594 anti-rabbit IgG	Invitrogen, UK	goat	IF	1:500

Table 2.4 Secondary antibodies used in VM cultures and their respective dilutions and details.
Immunoperoxidase (IP), immunofluorescence (IF).

2.4 *In-vivo* methods

The LPS model of nigral dopaminergic cell death was used to investigate neuroprotective properties of OPN in rats. LPS produces dopaminergic cell loss in the SN via acute activation of glial cells and producing an inflammatory reaction at the SN (Herrera *et al.*, 2000; Iravani *et al.*, 2005). Details of the methods used are decribed below.

2.5 Animals

Adult male Wistar rats (250-350g, Harlan, UK) were housed in pairs according to standard conditions (21-24˚C, 55-65% humidity with a 12hr light/dark cycle) and had free access to pelleted diet and water. All experiments were performed under Animal (Scientific procedures) act 1986 and approved by King's College Ethical Review panel, Project licence 70/6018.

2.5.1 The LPS model of inflammation in PD

Male Wistar rats (250-300g) were anaesthetised with isoflorane (4% in 5%O_2: 95% CO_2) and secured in stereotaxic frame (Kopf instruments, US). The incisor bar was positioned 3.3 mm below the interaural line. Anaesthesia was maintained using isoflorane (1.5-2%) and body temperature was maintained at 37˚C using a homeothermic blanket (Harvard instruments, US). In order to expose the skull, the scalp of the rat was shaved, sterilised with ethanol (70%) and a vertical cut was made along the midline using a sterile disposable scalpel (No. 11, Swan-Morton, UK). The bregma was located and immediately above SN a 1.5mm hole was drilled into the skull at co-ordinates -48mm anterior and +2.0mm lateral to bregma (Paxinos and Watson, 1986). A Hamilton syringe (70IRN; Scientific laboratory supplies, UK) (10µl) mounted on the stereotaxic frame was placed over the hole and the needle (26s; Hamilton, Scientific laboratory supplies, UK) was slowly lowered into the brain, 7.6mm below the dura into the SN. LPS (5µg/µl; 2µl) or vehicle (0.9% saline; 2µl) was slowly injected over 2min. The needle was left in-situ for a further 4min after injection before being slowly withdrawn. The skull was then swabbed with ethanol (70%) and the wound closed using absorbable sutures (Ethicon-coated vicryl, *Johnson's & Johnson's*, UK). After surgery, the rats were injected with a glucose solution to prevent dehydration (5ml i.p. of 5% glucose/0.9% sodium chloride). The rats were allowed to recover from anaesthesia before being returned to their home cages where they had access to mashed diet. All animals were weighed regularly and those that had lost 10% of their body weight were given mash until they start gaining weight. Figure 2.10 shows typical loss of dopaminergic neurons 7 days following LPS lesioning.

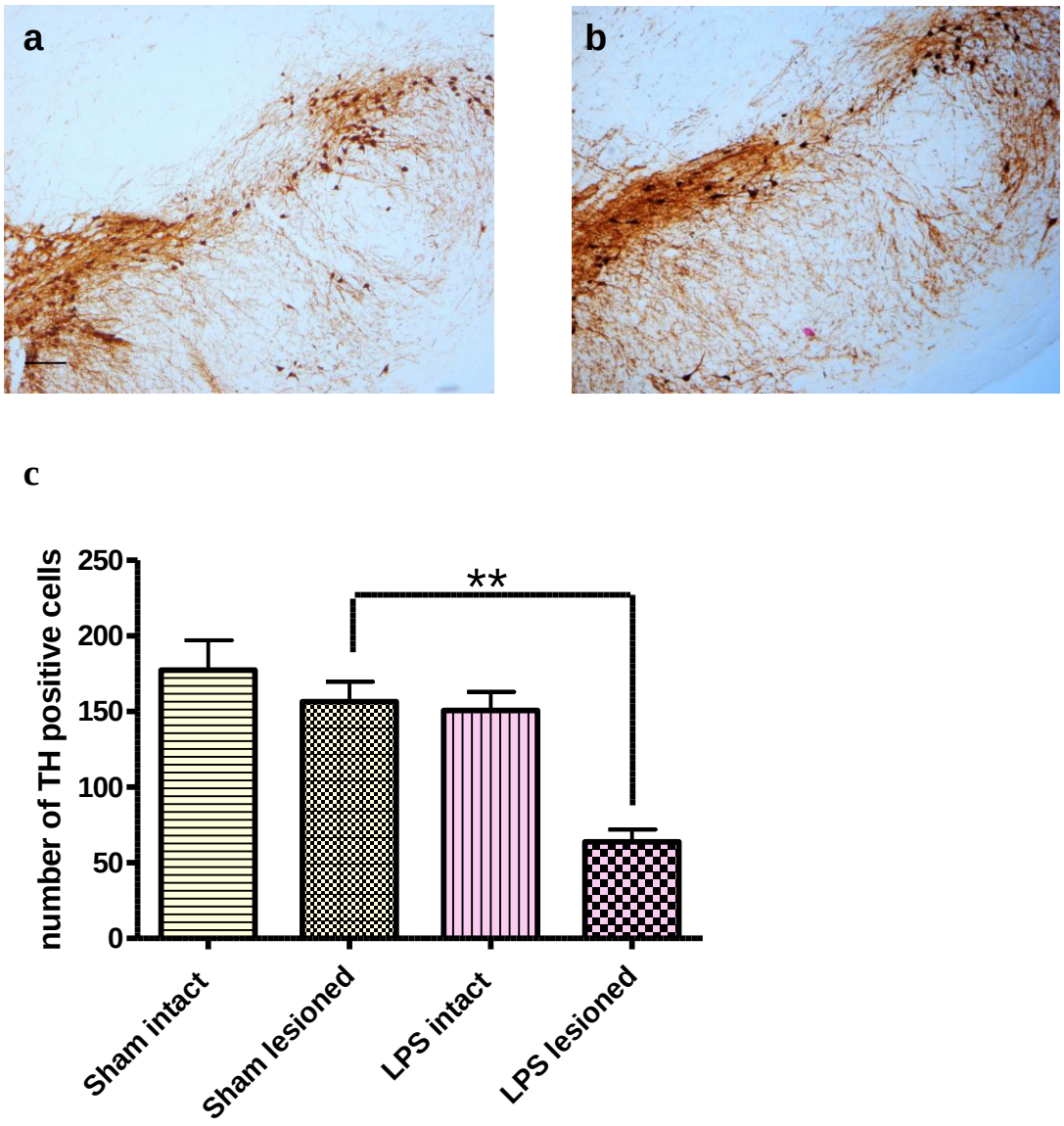

Figure 2.10 The number of TH positive cells in the SN one week following LPS intra-nigral injection.

(a) TH immunopositive cells in the sham lesioned SN. (b) TH immunopositive cells in the LPS lesioned SN. (c) TH cell counts in the SN comparing the sham lesioned and LPS lesioned SN (n=3-6/group). Magnification x5, scale bar = 400μm and is representative of both images. **P<0.01 (one-way ANOVA followed by Newman Keuls test).

2.5.2 Immunohistochemistry in rat brain tissue

2.5.2.a Tissue preparation

Animals were deeply anaesthetised using pentobarbital (130mg/kg i.p.). The thoracic cavity was exposed and animals were transcaridally perfused through the left ventricle with ice cold 0.1M PBS (0.1M; 100ml per animal). Subsequently, brains were removed, post fixed in PFA (4%/0.1M PBS) for five days then cryoprotected in sucrose (30% in 0.1M PBS with 0.05% sodium azide) and kept at 4˚C until use.

When the tissue had equilibrated, it was mounted on a sliding microtome (Leica, Germany) and sectioned coronally through the SN according to the rat brain atlas of Paxinos and Watson (1986). Free-floating sections of 30µm thickness were collected in 24 well plates filled with cold PBS (0.1M) with sodium azide (0.05%) for long term storage at 4˚C.

2.5.2.b Peroxidase immunohistochemistry in free-floating sections

This technique was carried out in 24 well plates (1ml/well). Sections were incubated for 30min in H_2O_2 (0.3%/0.1M PBS) to block endogenous peroxidises. Non specific binding was blocked by incubating the sections with 20% blocking solution (20% goat serum, 0.1% Triton-X 100/0.1M PBS) for one hour. After two 10min washes with PBS (0.1M), the sections were incubated with primary antibodies (Table 2.5) diluted in 1% blocking solution (1% goat serum, 0.1% Triton-X 100/0.1M PBS) overnight at room temperature. After two 10min washes in 0.1M PBS, sections were incubated with biotinylated secondary antibodies (Table 2.6) diluted in 0.05%Triton-X/0.1M PBS for one hour. The sections were washed twice (10min) in 0.1M PBS followed by incubation in the Avidin Biotin complex (1:200) for one hour then washed twice in Tris-HCL (0.05M). For visualisation of results, sections were incubated with DAB (0.05% in 0.05M Tris-HCL; Ph 7.4) for 5min then H_2O_2 (0.01% final concentration) was added and the sections incubated for 2-5min. The reaction was terminated by removing DAB and washing sections three times (10min) in Tris-HCL (0.05M). Sections were mounted onto polysine slides and dried overnight. Finally, slides with mounted sections were dehydrated through series of ethanol solutions (70%, 96%, 100%), cleared in histoclear (15min) and coverslipped with glass coverslips (22x50cm; VWR,UK) using DPX mounting

medium. A Zeiss Axioskop 40 microscope was used to examine the sections. An example of typical results obtained for GFAP immunoperoxidase staining in the brain of a normal rat is shown in Figure 2.11.

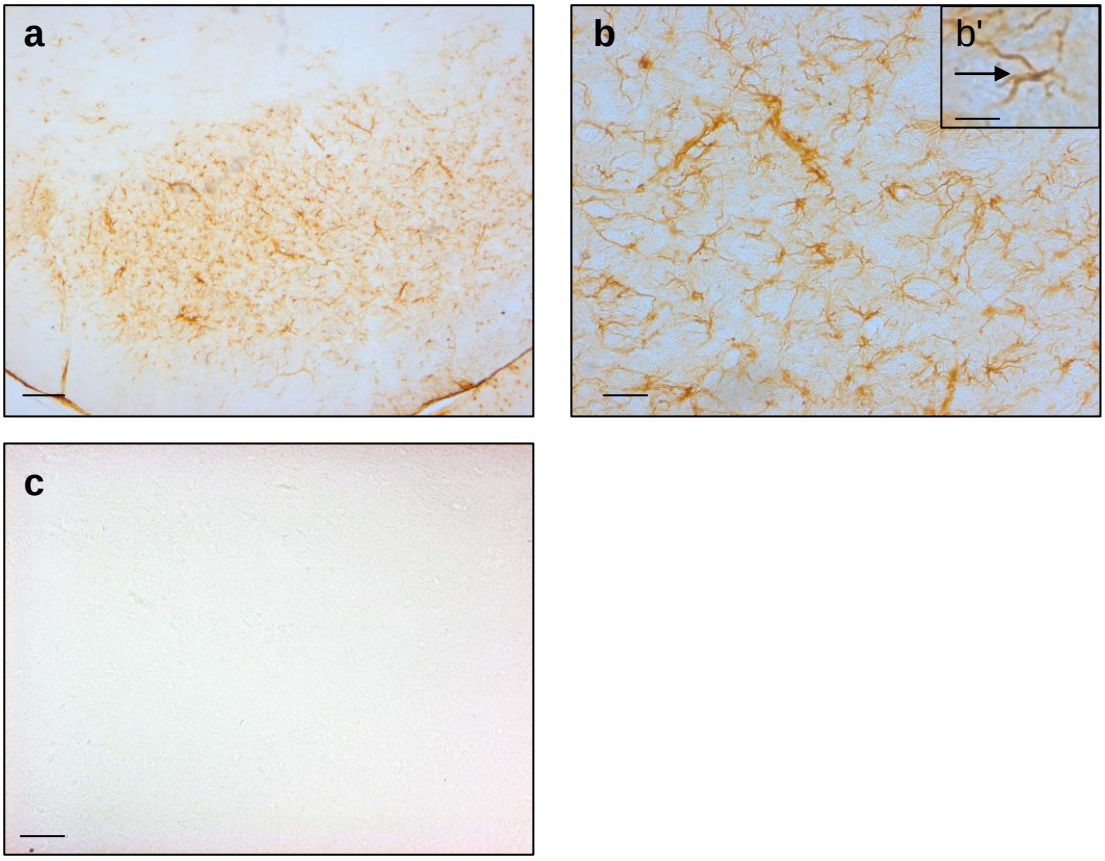

Figure 2.11 Typical GFAP immunoperoxidase staining in the rat SN.

(a,b) GFAP immunostaining in the SN of a naive rat brain. Magnifications x5 and x20 respectively, scale bars = 200µm, 100µmrespectively. Insert in (b), → GFAP positive cell. Magnification x100, scale bar = 10µm. (c) Primary antibody omitted, biotinylated anti-mouse secondary antibody used. Magnification x20, scale bar = 100µm.

2.5.2.c Quantification of positive staining

Cell counts were performed for the following markers: TH, OX-6 and ED-1. Three SN tissue sections from each animal were viewed using Zeiss Axioskop 40 microscope at magnification x20 and cell counts performed manually in a blind manner. For TH, immunopositive cells were counted from the whole SNpc but for OX-6 and ED-1, immunopositive cells were counted in five random areas of the SN using an eye piece grid (total area =1.25mm^2). For GFAP and receptor staining

images were taken using an Olympus BX-61 microscope at magnification x1.25, then optical density (OD) of staining in the SN measured using Image J software.

2.5.2.d Double immunofluorescence staining in SN tissue sections

This technique was carried out in 24 well plates (1ml/well). SN sections were incubated for an hour with 10% blocking solution (5% goat serum, 5% donkey serum, 0.05% Triton-X 100 in 0.1M PBS). Then, sections were incubated with two different primary antibodies raised in different species (Table 2.5) diluted in 1% blocking solution (0.5% goat serum, 0.5% donkey serum, 0.05% Triton-X 100 in 0.1M PBS) overnight at 4˚C. On the next day, after two 10min washes with PBS (0.1M), cells were incubated with the relevant secondary antibodies (Table 2.6) conjugated with Alexa Fluor 594 (red) or Alexa Fluor 488 (green) for 2hr in a darkened environment. After three 30min washes with PBS (0.1M), coverslips were mounted, in the dark, onto polysine slides using vectashield hardset mounting medium with DAPI. A Zeiss Axioskop 40 microscope was used to examine the sections. Specificity of antibodies was tested by omitting the primary antibody. Figure 2.12 shows an example of double immunofluorescence staining in the SN.

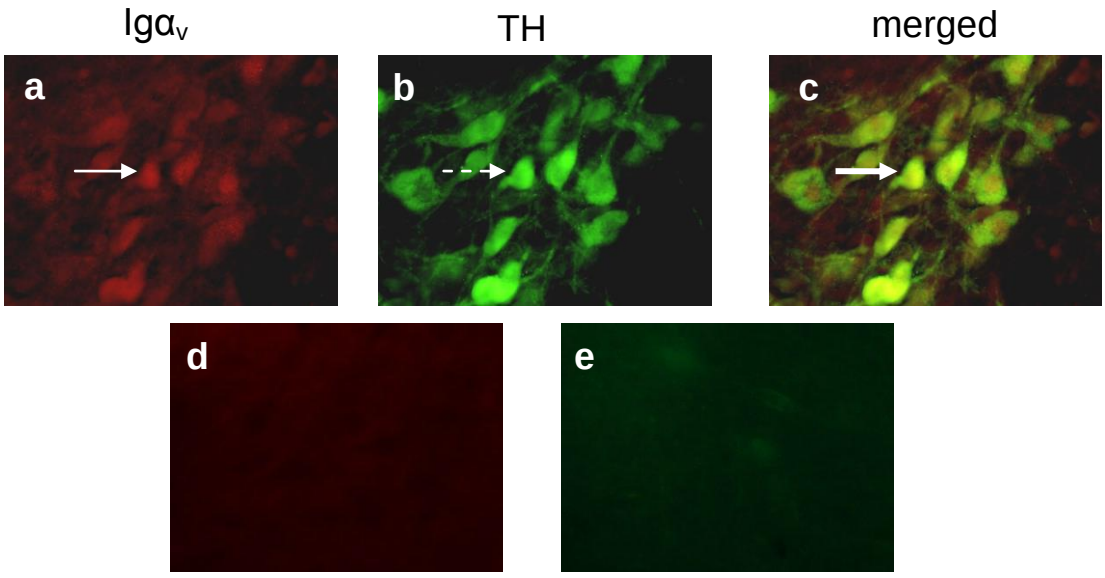

Figure 2.12 Typical double immunofluorescence labelling of TH and Igα$_v$ in the SN.

(a-c) Igα$_v$ positive cells are stained in red (→) and TH positive cells are stained in green (- - →). The merged picture (c) shows co-localisation between TH and Igα$_v$ (→). (d-e) Primary antibody omitted, (a) Alexafluor 594 anti-rabbit secondary antibody and (b) fluorescin conjugated anti-mouse antibody used. Magnification x40, scale bar= 40µm and is representative of all images.

Antibody	Supplier	Species	Dilution (IP)	Dilution (IF)	Antigen
Anti-TH	Pelfreeze, USA	rabbit	1:500	1:200	TH
Anti-TH	Sigma-Aldrich, UK	mouse	1:500	1:200	TH
Anti-GFAP	Merck bioscience, UK	mouse	1:500	1:200	Glial fibrillary acidic protein
Anti-OX-42	AbD Serotec, UK	mouse	1:200	-	CR3 receptor on microglia, macrophages
Anti-OX-6	AbD Serotec, UK	mouse	1:200	1:100	MHC Class II Ia receptor on microglia, macrophages
Anti-NeuN	Millipore, UK	mouse	1:200	1:100	NeuN
Anti-ED-1	AbD Serotec, UK	mouse	1:500	1:200	Macrophages
Anti-OPN	Hybridoma bank, Canada	mouse	1:500	1:200	OPN
Anti-Igα_v	Santa cruz biotechnology, Germany	rabbit	1:100	1:50	Igα_v receptor
Anti-Igβ_3	Santa cruz biotechnology, Germany	rabbit	1:50	1:25	Igβ_3 receptor
Anti-Igβ_1	Santa cruz biotechnology, Germany	rabbit	1:50	1:25	Igβ_1 receptor
Anti-CD44	Abcam, UK	rabbit	1:100	1:50	CD44 receptor

Table 2.5 Primary antibodies used *in-vivo* with their respective details and dilutions.

Immunoperoxidase (IP), immunofluorescence (IF).

Antibody	Supplier	Species	Technique	Dilution
Biotynilated anti-rabbit IgG	Vector Laboratories, UK	goat	IP	1:200
Biotin conjugated anti-mouse IgG	Jackson Immunolabs, UK	goat	IP	1:200
Biotynilated anti-mouse IgG	Vector Laboratories, UK	goat	IP	1:200
Alexa Fluor 488 anti-mouse IgG	Invitrogen, UK	donkey	IF	1:200
Alexa Fluor 954 anti-rabbit IgG	Invitrogen, UK	goat	IF	1:500

Table 2.6 Secondary antibodies used *in-vivo* with their respective details and dilutions.

Immunoperoxidase (IP), immunofluorescence (IF).

Reagents	Source
30% Acrylamide/bis soulution, 37.5:1	Bio-Rad, Hertfordshire, UK
Ampicillin	VWR international, Lutterworth, UK
Cell titre glo Luminescent cell viability assay	Promega, Southampton, UK
Cryospray	Bright instrument CO Ltd.Cambridgeshire, UK
Cytot Tox-ONE TM Homogenous membrane integrity assay	Promega, Southampton, UK
DPX mounting medium	BDH, VWR international, Lutterworth, UK
Dulecco's modified eagle medium(DMEM)-Glutamax	Invitrogen, Paisley, UK
Enhanced chemiluminescence (ECL) plus Blotting detection system	Amersham Biosciences, Buckinghamshire, UK
EmlaTM Cream	BSU, KCL, UK
F- 12k nutrient mixture, kaighn's medium	Invitrogen, Paisley, UK
Foetal bovine serum	Invitrogen, Paisley, UK
Horse serum	Invitrogen, Paisley, UK
G-418- Sulphate solution	PAA laboratories Ltd., Yeovil, UK
GFP fusion topo expression kit	Invitrogen, Paisley, UK
Histoclear	Fisher Scientific, Leicestershire, UK
Isofluorane	BSU, KCL, UK
Iso-Pentane	BDH, VWR international, Lutterworth, UK
Kodak hyperfilm	GE Healthcare Ltd., Buckinghamshire, UK
FuGENE 6 transfection reagent	Roche, Burgess Hill, UK
Methanol	BDH, VWR international, Lutterworth,UK
MG-132	Calbiochem, Nottingham, UK
LPS	Calbiochem, Nottingham, UK
Plasmid Midi Kit	Qiagen, Crawley, UK

Precision Plus protein standard	Bio-Rad, Hertfordshire, UK
Proteasome inhibitor cocktail set III	Calbiochem, Nottingham, UK
PSN	Invitrogen, Paisley, UK
PVDF membrane	Bio-Rad, Hertfordshire, UK
QIAprep Spin Mini kit	Qiagen, Crawley, UK
RNeasy Mini Kit	Qiagen, Crawley, UK
Sodium pentobarbitone (Euthatal®)	Merial, Dundee, UK
Standard Avidin biotin complex (ABC)	Vector laboratories, Peterborough, UK
Sterile water	PAA laboratories Ltd., Yeovil, UK
Sucrose	Merck Biosciences Ltd., Nottingham,UK
Tris-(hydroxymethyl) methylamonium chloride (Tris-HCL)	BDH, VWR international, Lutterworth,UK
Trypsin (1x)	Invitrogen, Paisley, UK
Vectashield® Hard Set mounting medium (with/without Dapi)	Vector laboratories, Peterborough, UK

Table 2.7 List of chemicals, drugs and reagents used.

Chemicals and reagents not listed in this Table were all obtained from Sigma-Aldrich, Dorset, UK.

Chapter 3 OPN does not prevent toxin induced cell death in dopaminergic neuronal cell lines despite the presence of integrin and CD44 receptors

3.1 Introduction

OPN possesses many of the properties required for an effective neuroprotective agent in PD including anti-inflammatory, anti-apoptotic and anti-oxidant properties (Chapter 1). Protective actions of OPN have been demonstrated in models of other diseases ranging from stroke to renal injury and these effects are thought to be related to actions of the protein on integrin and CD44 receptors (Chapter 1).

Particularly relevant to PD, OPN is present in the SN of normal rats, marmosets and in man (Iczkiewicz *et al.*, 2004). Importantly, levels of OPN are decreased in remaining dopaminergic neurones of the SN in PD compared to age-matched controls and following MPTP challenge in marmosets (Iczkiewicz *et al.*, 2006), suggesting that this protein could be intrinsically protective. The protective properties of OPN are decreased in aged mice compared to younger animals (Rollo *et al.*, 1996a) and this has significance to the age dependent prevalence of PD. When the current studies were started, it was not known whether OPN possesses protective properties against injury to dopaminergic cells in culture or whether endogenous OPN imports protective effects, or influences the action of exogenous OPN.

The key question is whether endogenous OPN expression is sufficient to significantly alter the sensitivity of neurons to toxic insult and whether exogenous OPN can exert any additional effect in protecting cells. A further significant question is whether the protective effects of OPN are mediated through integrin or CD44 receptors located on dopaminergic cells. It is known that full length OPN can bind to a number of integrin receptors notably $Ig\alpha_v$, $Ig\beta_3$, $Ig\beta_1$ as well as the CD44 receptor and that these are involved with cell survival actions of the protein (Caers *et al.*, 2006; Lee *et al.*, 2007; Lin *et al.*, 2001; Scatena *et al.*, 1998).

A range of dopaminergic cell lines are available, and these potentially offer a means of answering these questions but whether they differ in basal OPN expression has not been investigated. These cell lines can be used to manipulate OPN expression through transfection and subsequently to determine the effects of introducing OPN expression in an OPN null cell line. Cell lines are the simplest system to start this investigation.

Therefore, in these studies, a variety of cell lines commonly used in PD research (SH-SY5Y, SK-NMC, N1E-115, PC-12 and NTera-2) were used to

investigate the protective properties of full length OPN and whether these are mediated via the receptors previously shown to mediate pro-survival actions of the protein ($Ig\alpha_v$, $Ig\beta_3$, $Ig\beta_1$ and CD44). Cell lines were characterised for OPN expression and cells not normally expressing OPN were transfected to express the protein. Then, the effects of endogenous expression of OPN or exogenous treatment with OPN on the toxic effects of MPP^+ and H_2O_2 were investigated.

3.1.1 Hypothesis

It was hypothesized that:

- Endogenous OPN expression is neuroprotective and its absence renders cells more sensitive to toxic insult.
- Exogenous OPN treatment is protective in toxin treated cell lines because of the presence of $Ig\alpha_v$, $Ig\beta_3$, $Ig\beta_1$ and CD44 receptors.

3.1.2 Aims

In order to test the hypothesis, the potential protective properties of OPN were investigated in: cell lines endogenously expressing OPN, cells not expressing it and cells transfected with OPN. Detailed aims of the studies were:

- To characterise SH-SY5Y, SK-NMC, N1E-115, PC-12 and NTera-2 cell lines for their dopaminergic phenotype.
- To characterise SH-SY5Y, SK-NMC, N1E-115, PC-12 and NTera-2 cell lines for their OPN, $Ig\alpha_v$, $Ig\beta_3$, $Ig\beta_1$ and CD44 expression profile.
- To determine the susceptibility of OPN-null and OPN-positive (SH-SY5Y, N1E-115 and SH-SY5Y-OPN^+) cells to toxin-induced cell death.
- To investigate the effect of exogenous OPN treatment on toxin-induced cell death in OPN-null and OPN-positive cells (SH-SY5Y, N1E-115 and SH-SY5Y-OPN^+).
- To investigate whether protective effects of OPN are mediated via $Ig\alpha_v$, $Ig\beta_3$, $Ig\beta_1$ or CD44 receptors.

3.2 Methods

Cell lines represent an important method for studying neuroprotective agents and investigating mechanistic aspects. They allow performance of many manipulations such as gene over-expression or silencing, investigating pharmacological effects of inhibitors and agonists or testing fragments of the protein for activity relatively simply. Neuronal cell lines were used in this chapter to produce *in-vitro* models of dopaminergic cell death using the non-specific toxin that works by inducing oxidative stress H_2O_2 and the mitochondrial complex I inhibitor MPP^+. Methodology used to achieve the aims of this chapter is detailed below.

3.2.1 Cell lines

Five different cell lines, SH-SY5Y (human neuroblastoma), SK-NMC (human neuroblastoma), N1E-115 (mouse neuroblastoma), PC-12 (rat pheochromocytoma) and Ntera-2 (human embryonal carcinoma) were grown as described in Section 2.2.1 They were initially characterised for their TH, OPN and $Ig\alpha_v$, $Ig\beta_3$, $Ig\beta_3$ and CD44 receptors phenotype, before assessing neuroprotective properties of OPN.

3.2.2 Immunofluorescence

Immunofluorescence staining was used to characterise five cell lines (SH-SY5Y, SK-NMC, N1E-115, Ntera-2 and PC-12) for their TH, OPN, $Ig\alpha_v$, $Ig\beta_3$, $Ig\beta_3$ and CD44 receptors phenotype as described in detail in Section 2.2.6. Briefly, cells were incubated with blocking solution (20% Goat serum, 0.05% Triton-X100/ 0.1M PBS) for one hour then incubated with primary antibodies (Table 2.1) diluted in 1% goat serum/0.05% Triton-X100/0.1M PBS overnight at 4˚C. After two washes in 0.1M PBS, cells were incubated with the appropriate secondary antibodies (Table 2.2) diluted in 0.1M PBS for one hour at room temperature. A Zeiss Axioskop microscope was used to examine the cells.

3.2.3 Western blotting

Western blotting was performed in order to confirm the OPN phenotype of cell lines as described in Section 2.2.7. Briefly, aliquots of cell lysates (15µl) containing 100µg protein were loaded on SDS gel (12.5%). After electrophoresis, proteins were transferred from the gel into a PVDF membrane. Membranes were incubated in blocking solution (5% non-fat dry powder milk in TBS-T: Tris buffer 50mM pH 7.5, NaCl 150 mM, Tween 20 0.2%) overnight at 4˚C then incubated with mouse anti-OPN antibody (Hybridoma Bank; 1:3000) in blocking solution (2.5%) for three hours at room temperature. After three 10min washes in TBS-T, membranes were incubated for 1hr at room temperature with horse radish peroxidase conjugated goat anti-mouse antibody (1:2000) in blocking solution (2.5%). Bands were visualised as described in Section 2.2.7.

3.2.4 Transfection of SH-SY5Y cells with OPN DNA

SH-SY5Y cells which do not endogenously express OPN, were transfected with an OPN expressing vector or a control vector expressing green fluorescent protein (GFP).

3.2.4.a DNA cloning for transfection

Plasmid DNA designed with a human OPN insert (pEXP/pcDNA3.1/IOH13923; 1µl; Invitrogen) (Figure 3.1) or green fluorescent protein insert (GFP) (pcDNA3.1/NT-GFP; Invitrogen) (Figure 3.2) was incubated on ice with competent cells (XL1-Blue: Top10 strain; 20µl) for 30min. The cells were then heat-shocked in a water bath for 90sec at 42˚C to transform the cells then transferred back on ice for 2min. Luria-Bertani medium (80µl) was added to the mix and incubated at 37˚C for 45min. Transformation mix (8µl) was plated onto pre-warmed plates (37˚C) of Luria-Bertani agar with ampicillin (100µg/ml) and incubated overnight at 37˚C. The next day, small single clones were selected randomly and inoculated into Luria-Bertani medium (3ml; containing ampicillin, 100µg/ml) and incubated overnight at 37˚C on a shaking platform. Cells were harvested by centrifugation (2700g for 5min) and the DNA purified using Qiagen Medi-prep kit by following manufacturer instructions. A sample of the resulting

DNA was sent for sequencing at the molecular biology unit, King's College London where the sequences of human OPN DNA and GFP were confirmed.

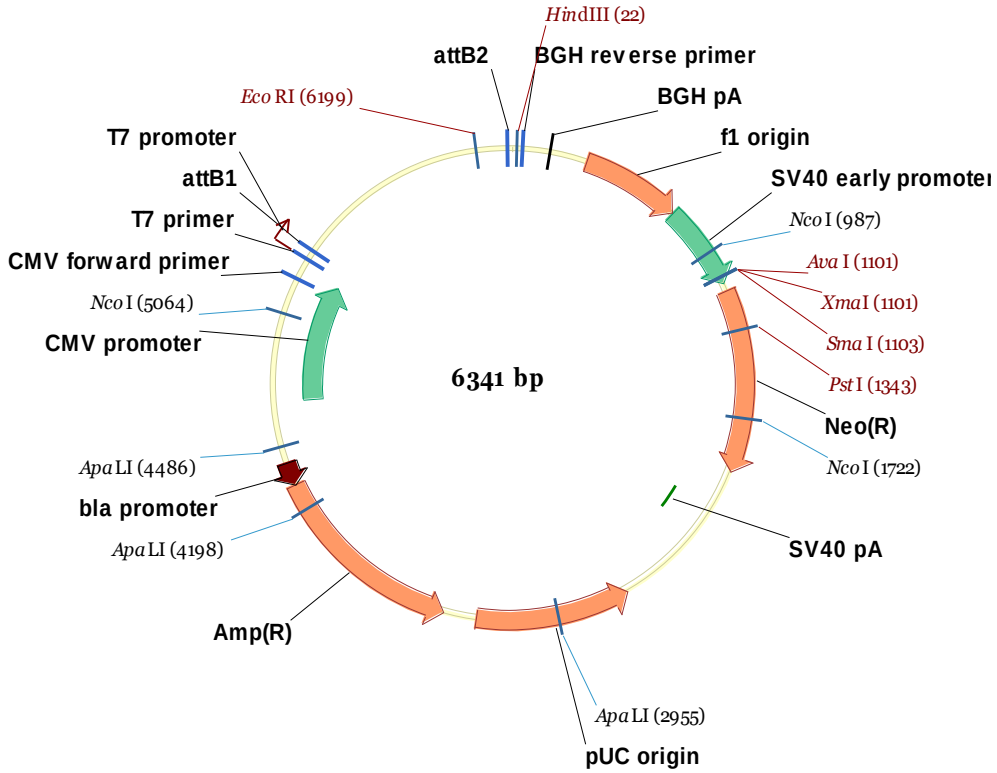

Figure 3.1 Schematic map of pcDNA3.1 vector with OPN insert (IOH13923) between gateway att sites, used for transfecting SH-SY5Y cells.

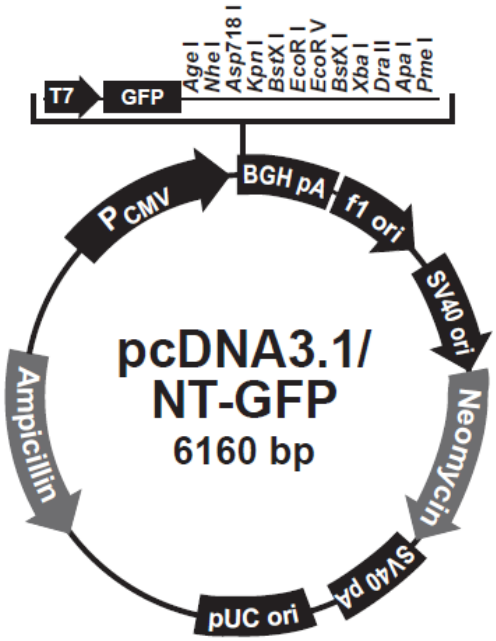

Figure 3.2 Schematic map of pcDNA3.1 vector with GFP insert, used for transfecting SH-SY5Y cells.

3.2.4.b Determination of the concentration of geneticin for selection

The vector used for this transfection (pcDNA3.1) confers geneticin resistance to the successfully transfected cells. Selection of transfected cells in all experiments was carried out over two weeks. In order to determine the geneticin concentration which kills naïve (non transfected) cells remaining after each transfection, the following experiment was carried out before starting transfection.

SH-SY5Y cells were plated in three 6 well plates at $2x10^5$cell per well, allowed to adhere overnight, then changed to media (DMEM-Glutamax, 10%FBS) containing geneticin (100-800µg/ml). Cells were grown for two weeks with geneticin media changed every two days. Using observation under the inverted microscope (Nikon VMS, UK), the smallest concentration of geneticin which caused total cell death in two weeks was found to be 300µg/ml (data not shown). This concentration was used for further transfection experiments.

3.2.4.c Optimisation of transfection conditions

Fugene 6 transfection reagent (Roche) was used according to manufacturer directions where it was mixed with OPN or GFP DNA in serum and antibiotic free media (DMEM-Glutamax) at three different concentration ratios (Fugene:DNA, 1:6; 2:6 and 4:6) and incubated for 15min. SH-SY5Y cells were seeded into 6 well plates at a fixed density ($4x10^5$ per well) as described in Section 2.2.1. After 24hrs, when the cells reached about 30% confluence, the transfection mix was added to the wells and spread by swirling the plates and left for 72hr to allow transfection of cells.

The cells were detached with trypsin and re-seeded into new 6 well plates at a density of $2x10^5$cell/well overnight then changed to selection media containing geneticin (300µg/ml), with selection media changed every two days to maintain geneticin concentration. Control non-transfected SH-SY5Y cells were grown in a separate 6 well plate in the same manner. After two weeks all the cells in the control (non-transfected) plate were dead, while transfected cells survived. When surviving transfected cells reached about 80% confluence the heterogeneous population was lysed and Western blotting performed to determine the expression of OPN as described in Section 2.2.7. The heterogeneous population obtained via transfecting conditions that resulted in the most expression of OPN (Fugene:DNA = 2:6) (Figure 3.3) was subsequently used.

3.2.4.d Selection of stably transfected SH-SY5Y clones

The transfection process was repeated as in Section 3.2.4.c using optimum transfecting conditions (Fugene:DNA = 2:6) and after 72hr exposure to DNA, cells were detached with trypsin, re-plated at $2x10^5$ on 6 well plates and grown in media containing geneticin (300µg/ml) until clones appeared. Clones were picked using cloning cylinders (Sigma) and transferred to single wells of 24 well plates. When clones reached confluence in the wells, they were transferred into separate T75 flasks and allowed to grow. Expression of OPN protein was confirmed by Western blotting and fluorescence immunocytochemistry (Figure 3.4). Control transfection was performed in the same manner using the GFP expressing vector and transfection was confirmed by immunofluorescence (Figure 3.4).

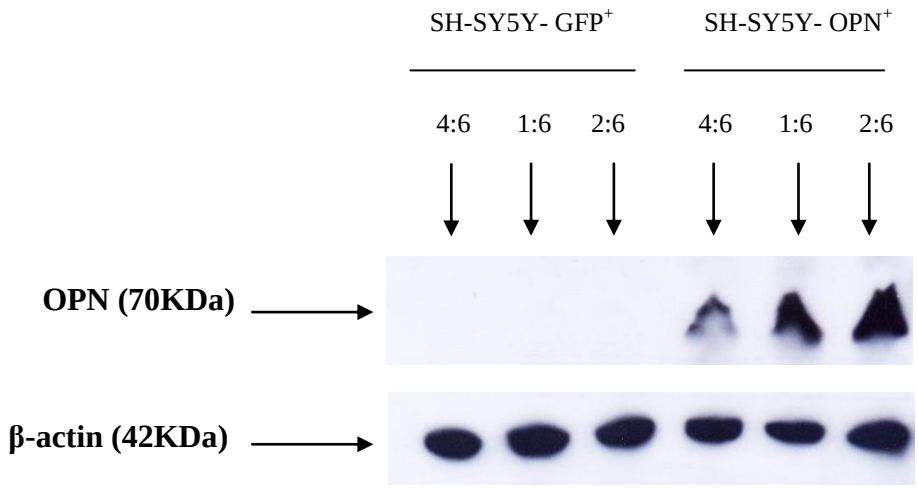

Figure 3.3 Typical Western blot showing OPN expression in different heterogeneous populations of OPN- transfected SH-SY5Y cells.

SH-SY5Y cells transfected with OPN or control (GFP) using different transfection conditions: three different concentration ratios of Fugene:DNA (1:6; 2:6 and 4:6) were lysed and separated on a 12% SDS PAGE and tested for OPN expression. Lysates were also tested for β-actin expression as a loading control.

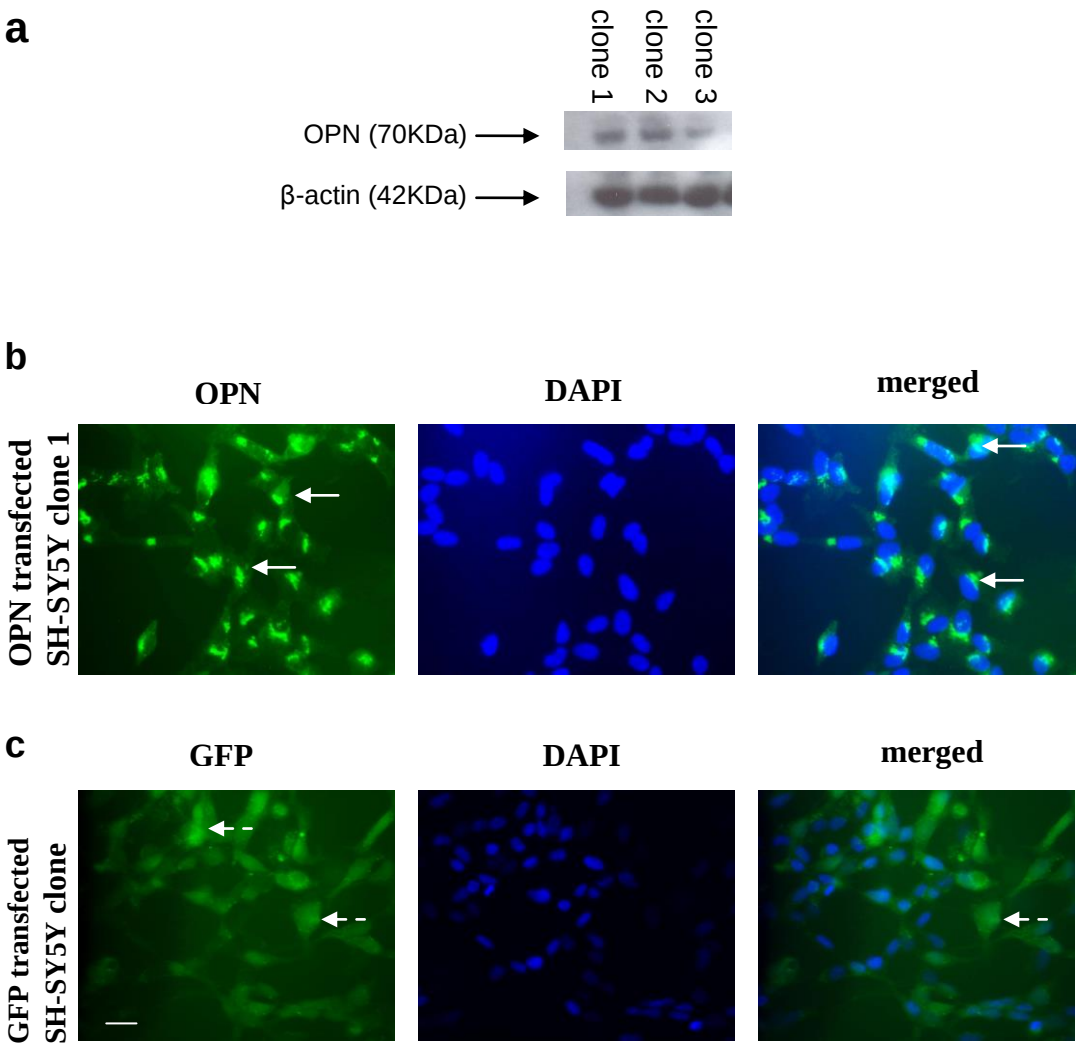

Figure 3.4 Characterisation of SH-SY5Y clones over-expressing OPN.

(a) Western blot analysis demonstrating over-expression of OPN protein in SH-SY5Y transfected clones. Clones 1-3 showed a positive band for OPN at 60KDa. β-actin was used as a loading control. (b-cc) Immunofluorescence confirmation of transfected clones. OPN transfected SH-SY5Y clone 1 shows positive immunoreactivity for OPN (b, →) and GFP transfected control clone shows green fluorescence from the expressed GFP protein (c, - →). Magnification x40, scale bar = 60µm and is representative of all images.

3.2.5 Determination of the EC_{50} concentration of MPP^+ and H_2O_2

Cells were plated into 96 well plates at 1-2 x 10^4cell/well in full growth medium (100µl/well) as described in Section 2.2.1. After 24hrs, medium was changed to serum free medium (DMEM-Glutamax supplemented with 100IU/ml penicillin and 100µg/ml streptomycin; 100µl) and after a further 24hrs treated with MPP^+ (0.1-10mM), H_2O_2 (50-800µM) or vehicle (serum free medium) by adding stock solutions (10µl; 10x the desired concentration) to each well (90µl). Cells treated with serum-free media only served as control. Twenty four hours after toxin treatment, cell death was measured by LDH and ATP assays and EC_{50} values were determined by non-linear regression analysis as described in detail in Section 2.2.8.c.

3.2.6 Neuroprotection assays

Cells were plated into 96 well plates at 1-2 x 10^4cell/well containing full growth medium (100µl/well) as described in Section 2.2.1. After 24hrs, medium was changed to serum free medium (90µl). In order to determine whether OPN exerted neuroprotective effects, the cells were pre-treated with OPN (1-1000ng/ml) or vehicle (serum free medium) 24hrs after plating by adding 10µl of stock solutions (10 times the desired concentration) to each well (90µl). Recombinant human OPN (R&D systems, UK) was used for human neuroblastoma cell line SH-SY5Y and recombinant mouse OPN (R&D systems, UK) was used for mouse neuroblastoma cell line N1E-115. After a further 24hr, cells were treated with MPP^+ or H_2O_2 at their pre-determined EC_{50} concentrations (Section 3.2.5). Control cells were incubated with OPN alone or toxin alone and untreated control cells were treated with serum free medium only. Twenty four hours after toxin insult, LDH and ATP assays were performed to measure cell death as described in Section 3.2.7.

3.2.7 Quantification of cell death

3.2.7.a LDH Assay

Cell death was estimated by measuring LDH leakage using the Membrane integrity assay (Promega). The assay was performed as per manufacturer protocol (Section 2.2.8a). Briefly, an aliquot of the assay mix (100µl) was added to each well and plates were shaken for 11sec then incubated at 37˚C for 10min. Stop solution

(50µl) was added to each well to stop the reaction then fluorescence was measured in a Gemini XS plate reader plate reader at 260/290nm.

3.2.7.b ATP Assay

Cell death was estimated using a different assay which gives a measure of the ATP content of cells (The Cell titre glo assay; Promega). The assay was used as per manufacturer protocol (Section 2.2.8b). Briefly, both cells and assay mix were equilibrated to room temperature for 30min then an aliquot of the assay mix (100µl) was added to each well and plates shaken rigorously for 2 min to induce cell lysis in order for the ATP to be released from cells. Luminescent signal was allowed to stabilise for 5min and luminescence read on a Gemini XS plate reader.

3.2.8 Statistical analysis

Assays were performed as triplicates on three to four occasions (n=3-4). Data obtained for determination of EC_{50} concentration of toxins were analysed by one-way ANOVA followed by Newman Keuls test. Data from neuroprotection studies were analysed by two-way ANOVA followed by Newman Keuls test. GraphPad Prism 5 was used to analyse the data.

3.3 Results

3.3.1 Characterisation of cell lines

Five cell lines; SH-SY5Y, SK-NMC, N1E-115, PC-12 and Ntera-2 were characterised for TH, OPN, CD44 receptor and Integrins Igα_v, Igβ_3, Igβ_1 expression by immunofluorescence staining.

3.3.1.a TH

All cell lines except Ntera-2 showed positive TH immunostaining; which was diffuse and cytoplasmic in nature (Figure 3.5). PC-12 and N1E-115 showed intense TH staining while SH-SY5Y and SK-NMC showed faint TH staining (Figure 3.5).

3.3.1.b OPN

Only N1E-115 and PC-12 cell lines expressed OPN protein, showing punctate cytoplasmic immunofluorescence staining (Figure 3.5). By contrast, SH-SY5Y, SK-NMC and Ntera-2 cells did not show positive immunoreactivity to OPN.

3.3.1.c Integrin and CD44 receptors

All cell lines expressed all integrin subunits investigated (Igα_v, Igβ_3, Igβ_1) (Figure 3.6). Integrin positive immunoreactivity appeared as punctate cytoplasmic staining. Intensity of integrin receptors staining was similar in all cell lines (Figure3.6).

All cell lines showed positive immunoreactivity for the CD44 receptor and this appeared as diffuse cytoplasmic staining (Figure 3.6). SH-SY5Y, SK-NMC, N1E-115 and Ntera-2 cells showed faint staining while PC-12 showed intense immunoreactivity (Figure 3.6).

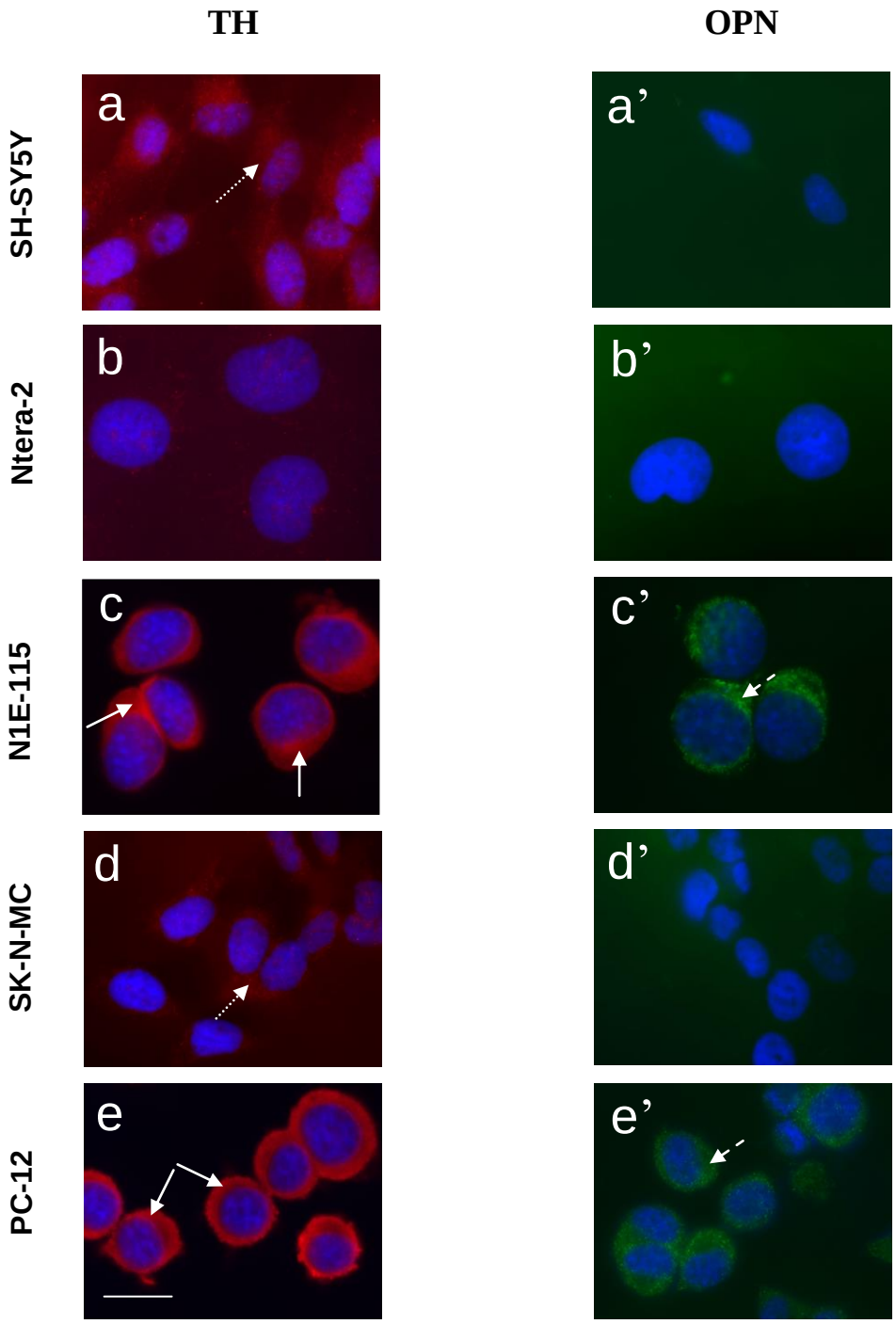

Figure 3.5 TH and OPN expression in SH-SY5Y, N1E-115, SK-NMC, PC-12 and Ntera-2 cell lines.

Representative photomicrographs showing TH immunoreactivity (red; a-e), OPN immunoreactivity (green; a'-e') and DAPI (blue) in SH-SY5Y, Ntera-2, N1E-115, SK-NMC and PC-12 cell lines respectively. ⟶ Intense TH staining, ⋯▸ faint TH staining, - -▸OPN staining. (Magnification x100, Scale bar = 10µm and is representative of all images).

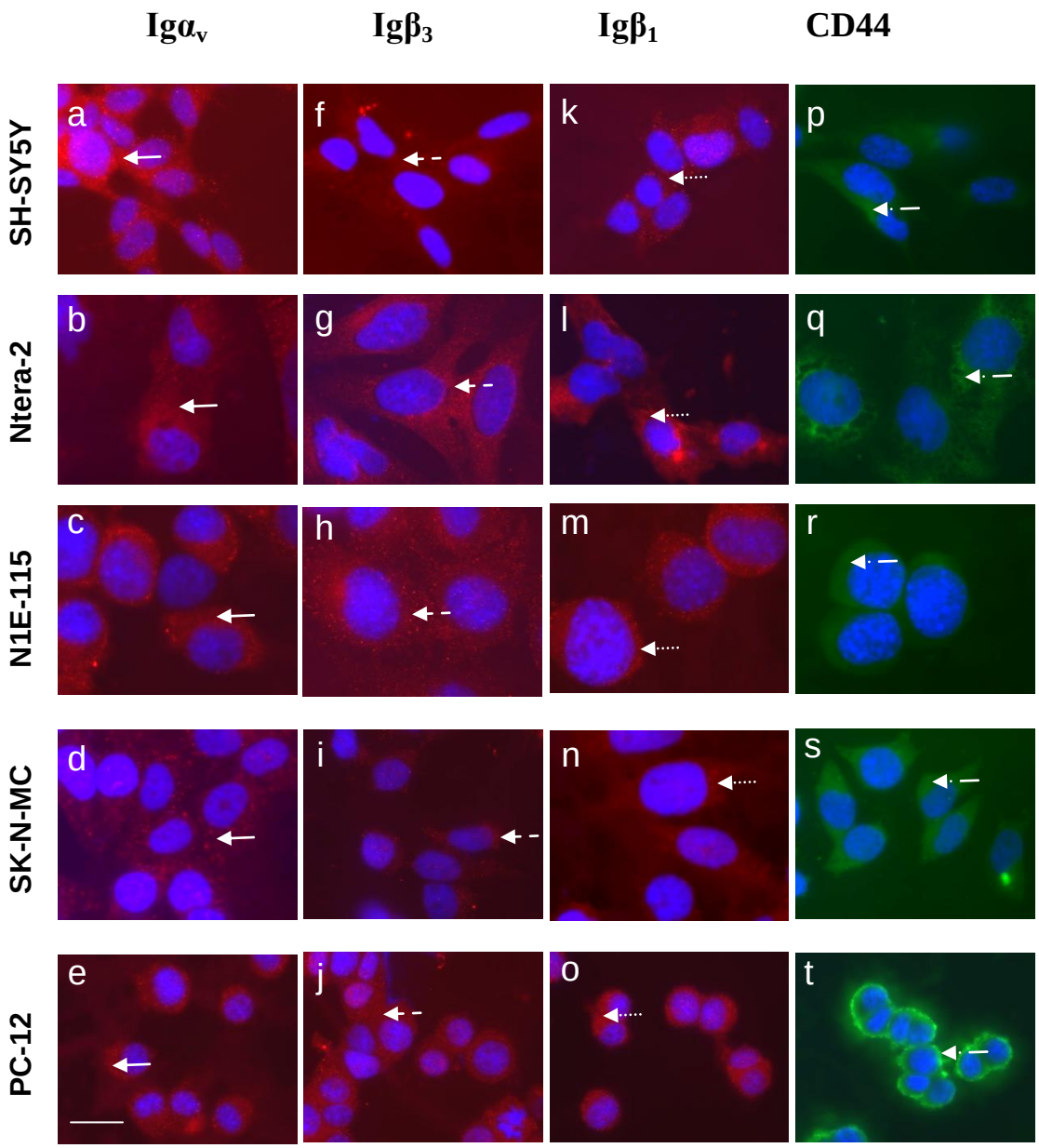

Figure 3.6 The expression of Igα$_v$, Igβ$_3$, Igβ$_1$ and CD44 receptors in SH-SY5Y, N1E-115, SK-NMC, PC-12 and Ntera-2 cell lines.

Representative photomicrographs showing integrin receptors immunoreactivity (red); Igα$_v$ (a-e, ⟶), Igβ$_3$ (f-j, - -▶), Igβ$_1$ (i-o, ····▶), CD44 immunoreactivity (green; p-t, —·▶) and DAPI (blue) in SH-SY5Y, Ntera-2, N1E-115, SK-NMC and PC-12 cell lines. Magnification x100 Scale bar = 10μm and is representative of all images.

	TH	OPN	Igα_v	Igβ_3	Igβ_1	CD44
SH-SY5Y	+	-	+	+	+	+
Ntera-2	-	-	+	+	+	+
N1E-115	+	+	+	+	+	+
SK-NMC	+	-	+	+	+	+
PC-12	+	+	+	+	+	+

Table 3.1 Summary of the expression of TH, OPN and receptors (Igαv, Igβ3, Igβ1 and CD44) in SH-SY5Y, Ntera-2, N1E-115, SK-NMC and PC-12 cells.

– represents no staining, + denotes positive staining.

3.3.2 Western blotting confirmation of OPN expression in cell lines

Expression of OPN was confirmed using Western blotting. N1E-115 and PC-12 cells expressed OPN protein while SH-SY5Y, SK-NMC, and Ntera-2 cells did not (Figure 3.7). This confirmed the results obtained by immunofluorescence staining (Section 3.3.1).

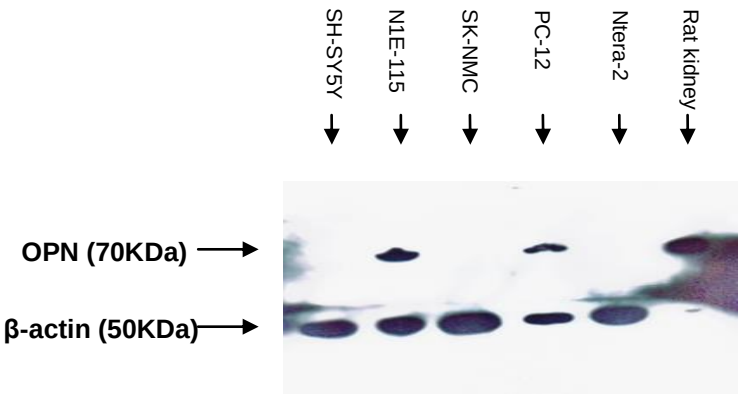

Figure 3.7 OPN expression in SH-SY5Y, N1E-115, SK-NMC, PC-12 and Ntera-2 cell lines.

Cell lysates of SH-SY5Y, N1E-115, SK-NMC, PC-12 and Ntera-2 were tested for OPN expression by Western blot. Rat kidney homogenate was used as a positive control. β-actin was used as a loading control.

3.3.3 Choice of cell lines

Cells were chosen based on four different criteria: expression of TH, OPN, OPN receptors and neuronal phenotype. First, in order for cell lines to be a suitable model of the vulnerable dopaminergic neurons which degenerate in PD, they should be neuronal and dopaminergic. Second, cells should contain the putative mechanism to respond to protective actions of OPN i.e. express receptors involved in the cell survival actions of OPN ($Ig\alpha_v$, $Ig\beta_3$, $Ig\beta_1$ and CD44) (Scatena *et al.*, 1998; Caers *et al.* 2006; Lin *et al*., 2000; Lee *et al*., 2007). In addition, the aim of this chapter was to determine whether endogenous OPN expression affected the ability of exogenously added OPN to protect them from toxin insults, hence different cell lines should differ in their OPN expression phenotype.

All cell lines except Ntera-2 cells were TH positive (Table 3.1) but only SH-SY5Y, SK-NMC and N1E-115 are neuronal. The five cell lines showed positive immunoreactivity to all receptors tested. However, they varied in their OPN expression profile with only N1E-115 and PC-12 cells showing positive expression (Table 3.1).

Two dopaminergic, neuronal cell lines; mouse neuroblastoma N1E-115 cells which *express* OPN endogenously and human neuroblastoma SH-SY5Y which *do not show positive expression* of OPN were chosen for further studies. SH-SY5Y cell line was chosen over SK-NMC as the former is more widely used in PD research and is more easily maintained.

In addition, SH-SY5Y cells were transfected with OPN DNA (SH-SY5Y-OPN^+) in order to investigate the effect of introducing expression of the protein, or with a control vector expressing GFP (SH-SY5Y-GFP^+). The transfection process induced higher growth rate in both SH-SY5Y-OPN^+ and control transfected SH-SY5Y-GFP^+.

3.3.4 The effect of OPN expression on the response to MPP^+

In order to establish the effect of OPN expression on MPP^+-induced cell death SH-SY5Y, N1E-115, SH-SY5Y-OPN^+ and SH-SY5Y-GFP^+ cells were treated with MPP^+ (0.1-10mM).

ATP assay

MPP^+ (0.1-10mM) induced a concentration-dependent increase in cell death in all cell lines as measured by the ATP assay (Figure 3.8). For SH-SY5Y and N1E-115 cells, cell death was observed between 1 and 10mM. Similarly, for SH-SY5Y-OPN^+ cell death was observed between 0.1 and 10mM and for SH-SY5Y-GFP^+ between 2 and 10mM (Figure 3.8). Maximal cell death of 100% was observed for SH-SY5Y, N1E-115 and SH-SY5Y-OPN^+ and 89% for SH-SY5Y-GFP^+ (Figure 3.8), although the latter value is not statistically different to that in the other cell lines (one-way ANOVA followed by Newman Keuls test).

The EC_{50} concentration of MPP^+ in SH-SY5Y-OPN^+ (2±0.1mM) was higher than that in SH-SY5Y (1.5±0.14mM) but the EC_{50} value of MPP^+ was also higher in control transfected SH-SY5Y-GFP^+ (3±0.4mM) and not different to SH-SY5Y-OPN^+ (Table 3.2). In addition, no significant difference was seen between EC_{50} values of MPP^+ in OPN expressing N1E-115 cells (2±0.22mM) or non OPN expressing SH-SY5Y cells (1.5±0.14mM) (Table 3.2).

LDH assay

MPP^+ (0.1-10mM) induced a concentration-dependent increase in cell death as measured by LDH leakage from cells (Figure 3.9). Basal LDH release was between 20-28% of lysis buffer control but was not significantly different in various cell lines (one-way ANOVA followed by Newman Keuls test).

For SH-SY5Y and N1E-115 cells, cell death was observed between 1 and 10mM. Similarly, for SH-SY5Y-OPN^+ and SH-SY5Y-GFP^+ cell death was observed between 0.1 and 10mM (Figure 3.9). Maximal cell death of 75%, 76%, 74% and 72% was observed for SH-SY5Y, N1E-115, SH-SY5Y-OPN^+ and SH-SY5Y-GFP^+ respectively (Figure 3.9), although value are not statistically different (one-way ANOVA followed by Newman Keuls test).

The LDH assay showed no difference in EC_{50} values of MPP^+ between OPN expressing N1E-115 cells (1±0.11mM) and non OPN expressing SH-SY5Y cells (2±0.33mM) (Table 3.2). By contrast, EC_{50} value of MPP^+ in SH-SY5Y-OPN^+ (3±0.02mM) was higher than that in SH-SY5Y and N1E-115 cells (Table 3.4). However, the EC_{50} value of MPP^+ was also higher in control trasfected SH-SY5Y-GFP^+ (3±0.2) and not different to SH-SY5Y-OPN^+ (Table 3.2).

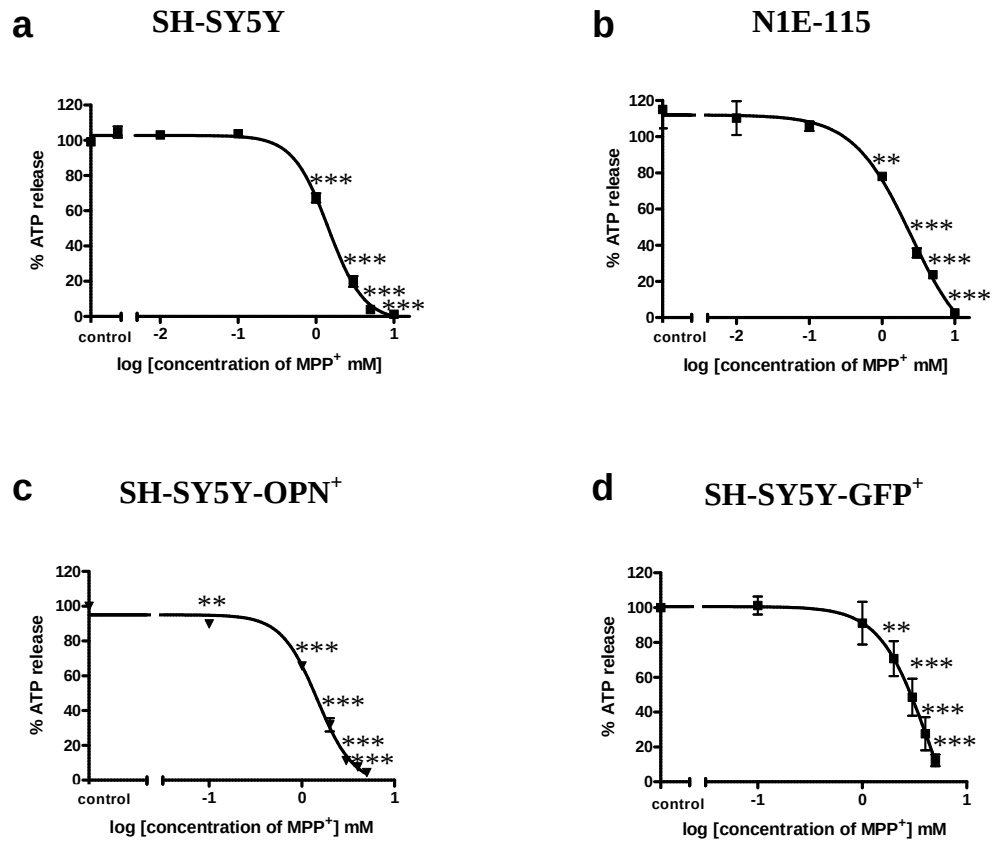

Figure 3.8 The effect of MPP^+ on ATP content in SH-SY5Y, N1E-115, SH-SY5Y-OPN^+ and SH-SY5Y-GFP^+ cells.

(a-d) Cells were treated with MPP^+ (0.1-10mM) for 24hr. Cell death was measured by ATP assay. Control untreated cells were taken as 100% ATP release. Data are expressed as mean ±SEM (n=3) and were analysed by non-linear regression. ** P <0.01, *** P <0.001 compared to control (one-way ANOVA followed by Newman Keuls test).

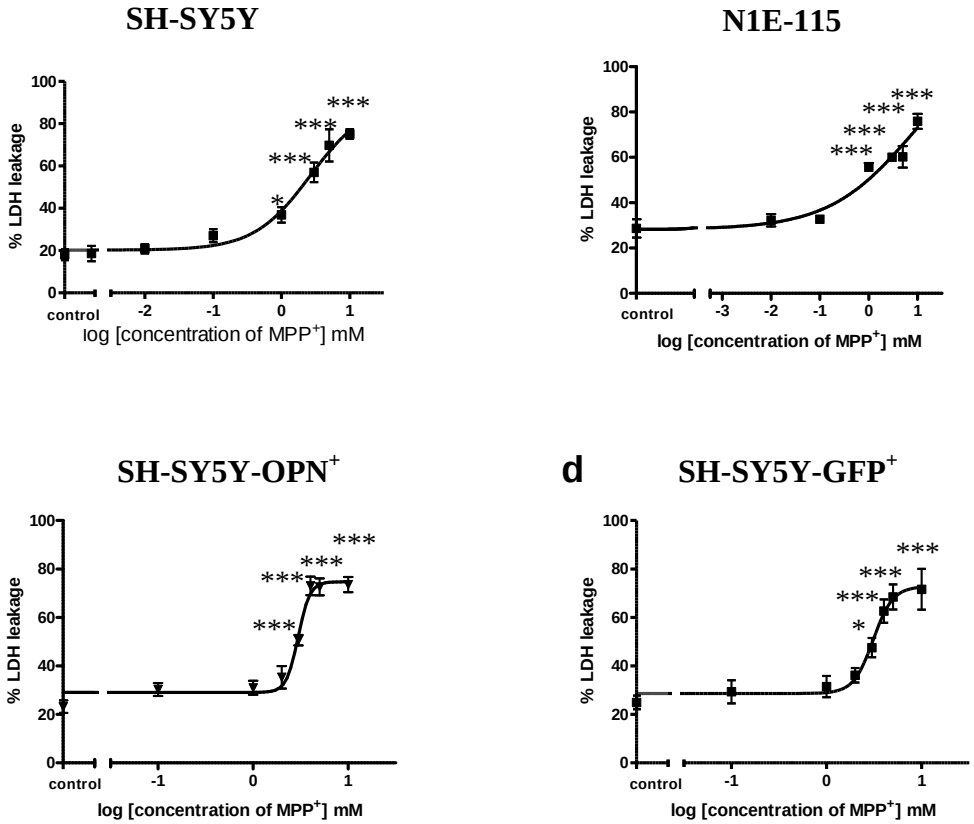

Figure 3.9 The effect of MPP^+ on LDH leakage in SH-SY5Y, N1E-115, SH-SY5Y-OPN^+ and SH-SY5Y-GFP^+ cells.

(a-d) Cells were treated with MPP^+ (0.1-10mM) for 24hr. Cell death was measured by LDH assay. Maximal LDH leakage from lysis buffer treated cells is 100%. Data are expressed as mean ±SEM (n=3) and were analysed by non-linear regression. * P <0.05, *** P <0.001 compared to control (one-way ANOVA followed by Newman Keuls test).

	SH-SY5Y	N1E-115	SH-SY5Y-OPN^+	SH-SY5Y-GFP^+
MPP^+ EC_{50} (mM) by ATP assay	* # 1.5±0.14	2±0.22	2±0.1	3±0.4
MPP^+ EC_{50} (mM) by LDH assay	** # 2±0.33	Δ § 1±0.11	3±0.02	3±0.2

Table 3.2 EC_{50} concentrations of MPP^+ in SH-SY5Y, N1E-115, SH-SY5Y-OPN^+ and SH-SY5Y-GFP^+ obtained by ATP and LDH assays.

Data are expressed as mean ±SEM (n=3). * P <0.05, compared to SH-SY5Y-OPN^+; ** P <0.01, compared to SH-SY5Y-OPN^+; # P<0.01 compared to SH-SY5Y-GFP^+; § P<0.001 compared to SH-SY5Y-OPN^+; Δ p<0.001 compared to SH-SY5Y-GFP^+ (one-way ANOVA followed by Newman Keuls test).

3.3.5 The effect of OPN expression on the response to H_2O_2

In order to establish the effect of OPN expression on toxin-induced cell death SH-SY5Y, N1E-115, SH-SY5Y-OPN$^+$ and SH-SY5Y-GFP$^+$ cells were treated with H_2O_2 (50-800µM).

ATP assay

H_2O_2 (50-800µM) induced a concentration-dependent increase in cell death in all cell lines as measured by the ATP assay (Figure 3.10). For SH-SY5Y cells, cell death was observed between 200 and 800µM H_2O_2 and N1E-115 between 50 and 800µM (Figure 3.10). Similarly, in SH-SY5Y-OPN$^+$ cell death was observed between 100 and 800 µM and in SH-SY5Y-GFP$^+$, between 200 and 800µM (Figure 3.10). Maximum cell death of 90%, 93%, 98% and 94% was measured at 800µM for SH-SY5Y, N1E-115, SH-SY5Y-OPN$^+$ and SH-SY5Y-GFP$^+$ respectively (Figure 3.10), although values were not statistically different (one-way ANOVA followed by Newman Keuls test).

The EC_{50} concentration of H_2O_2 in SH-SY5Y cells (417±29µM) was significantly higher than that in N1E-115 (268±44 µM) and SH-SY5Y-OPN$^+$ (361±44 µM) (Table 3.3). However, the EC_{50} concentration of H_2O_2 in SH-SY5Y-OPN$^+$ cells was not significantly different to that in SH-SY5Y-GFP$^+$ (352±25 µM).

LDH assay

H_2O_2 (50-800µM) caused a concentration-dependent increase in cell death in all cell lines as measured by LDH leakage from cells (Figure 3.11). Basal LDH release was between 15-26% (Figure 3.11) but was not statistically different in cell lines (one-way ANOVA followed by Newman Keuls test). H_2O_2 induced LDH leakage different to baseline at concentration 300µM in all cell lines (Figure 3.11).

In all cell lines, cell death was observed between 300 and 800µM (Figure 3.11). Maximum cell death of 71%, 85%, 100% and 96% was measured at 800µM for SH-SY5Y, N1E-115, SH-SY5Y-OPN$^+$ and SH-SY5Y-GFP$^+$ respectively (Figure 3.11). Maximum LDH leakage in transfected cells (SH-SY5Y-OPN$^+$ and SH-SY5Y-GFP$^+$) was higher than that in SH-SY5Y ($P<0.05$; one-way ANOVA followed by Newman Keuls test). There was no difference in EC_{50} values of H_2O_2 in all cells when measured by LDH assay (Table 3.3).

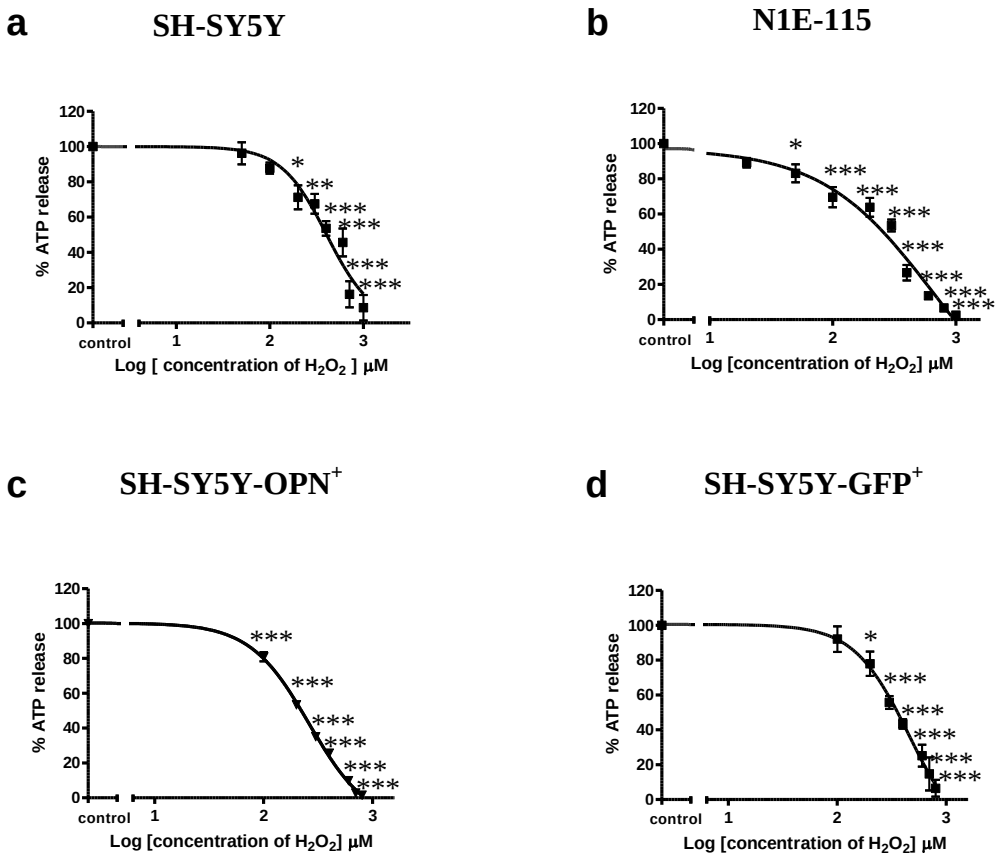

Figure 3.10 The effect of H_2O_2 on ATP content in SH-SY5Y, N1E-115, SH-SY5Y-OPN$^+$ and SH-SY5Y-GFP$^+$ cells.

(a-d) Cells were treated with H_2O_2 (50-800µM) for 24hr. Cell death was measured by ATP assay. Control untreated cells were taken as 100% ATP release. Data are expressed as mean ±SEM (n=3) and were analysed by non-linear regression. * P <0.05, ** P <0.01, *** P <0.001 compared to control (one-way ANOVA followed by Newman Keuls test).

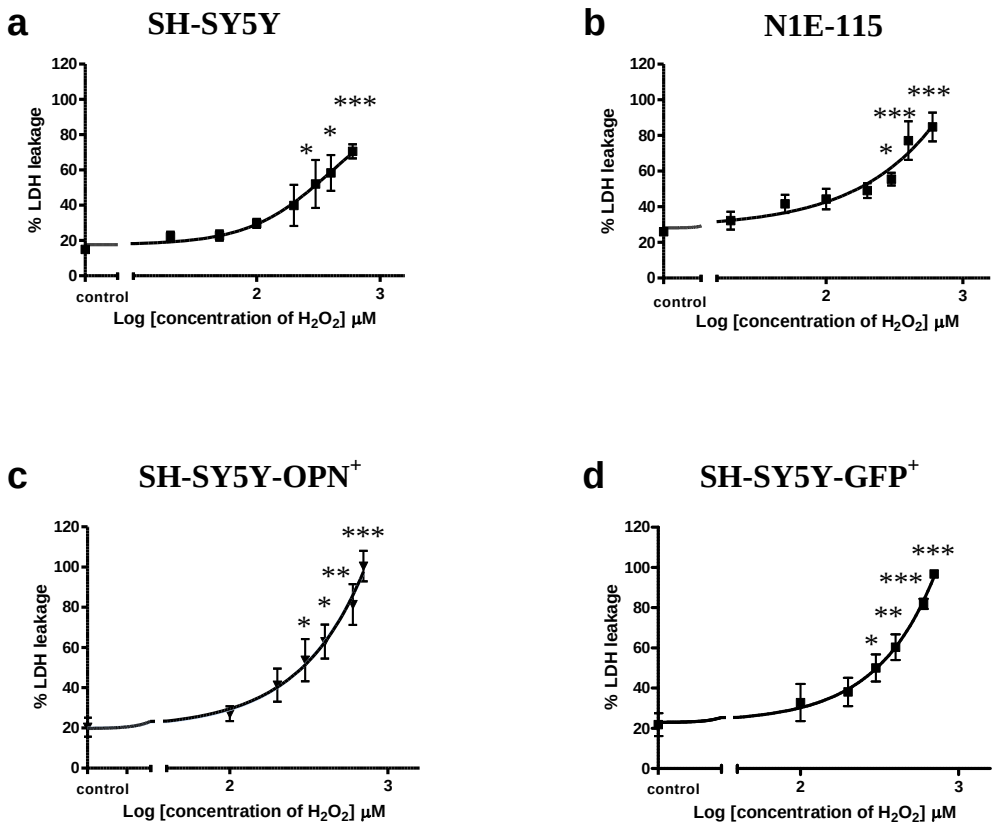

Figure 3.11 The effect of H_2O_2 on LDH leakage in SH-SY5Y, N1E-115, SH-SY5Y-OPN$^+$ and SH-SY5Y-GFP$^+$ cells.

(a-d) Cells were treated with H_2O_2 (50-800µM) for 24hr and cell death was measured by LDH assay. Maximal LDH leakage from lysis buffer treated cells is 100%. Data are expressed as mean ±SEM (n=3) and were analysed by non-linear regression. * P <0.05, ** P <0.01, *** P <0.001 compared to control (one-way ANOVA followed by Newman Keuls test).

	SH-SY5Y	N1E-115	SH-SY5Y-OPN$^+$	SH-SY5Y-GFP$^+$
H_2O_2 EC_{50}±SEM (µM) by ATP assay	# * 417±29	268±44	361±44	352±25
H_2O_2 EC_{50}±SEM (µM) by LDH assay	367±79	333±22	361±19	340±40

Table 3.3 EC_{50} concentrations of H_2O_2 in SH-SY5Y, N1E-115, SH-SY5Y-OPN$^+$ and SH-SY5Y-GFP$^+$ measured by ATP and LDH assays.

Data are expressed as mean ±SEM (n=3). *P <0.05 compared to N1E-115; # P <0.05 compared to SH-SY5Y-OPN$^+$ (one-way analysis of variance followed by Newman Keuls test).

3.3.6 The effect of OPN expression on the response to exogenous OPN treatment in MPP^+ treated cell lines

In order to determine the effect of OPN expression on the ability of OPN to protect against MPP^+-induced cell death, SH-SY5Y, N1E-115, SH-SY5Y-OPN^+ and SH-SY5Y-GFP^+ cells were pre-treated with OPN (1-1000ng/ml) in serum free medium or serum free medium alone prior to challenge with MPP^+ at pre-determined EC_{50} concentrations (Table 3.2). OPN treatment alone did not have any effect on cell viability of all cell lines (ATP and LDH assays) (Figures 3.12, 3.13). All toxins induced significant cell death (approximately 40-60%) as determined by LDH leakage and ATP content assays (Figures 3.12, 3.13). However, pre-treatment with OPN (1-1000ng/ml) did not have a protective effect against toxin-induced cell death in all cell lines regardless of the OPN expression phenotype (Figures 3.12, 3.13).

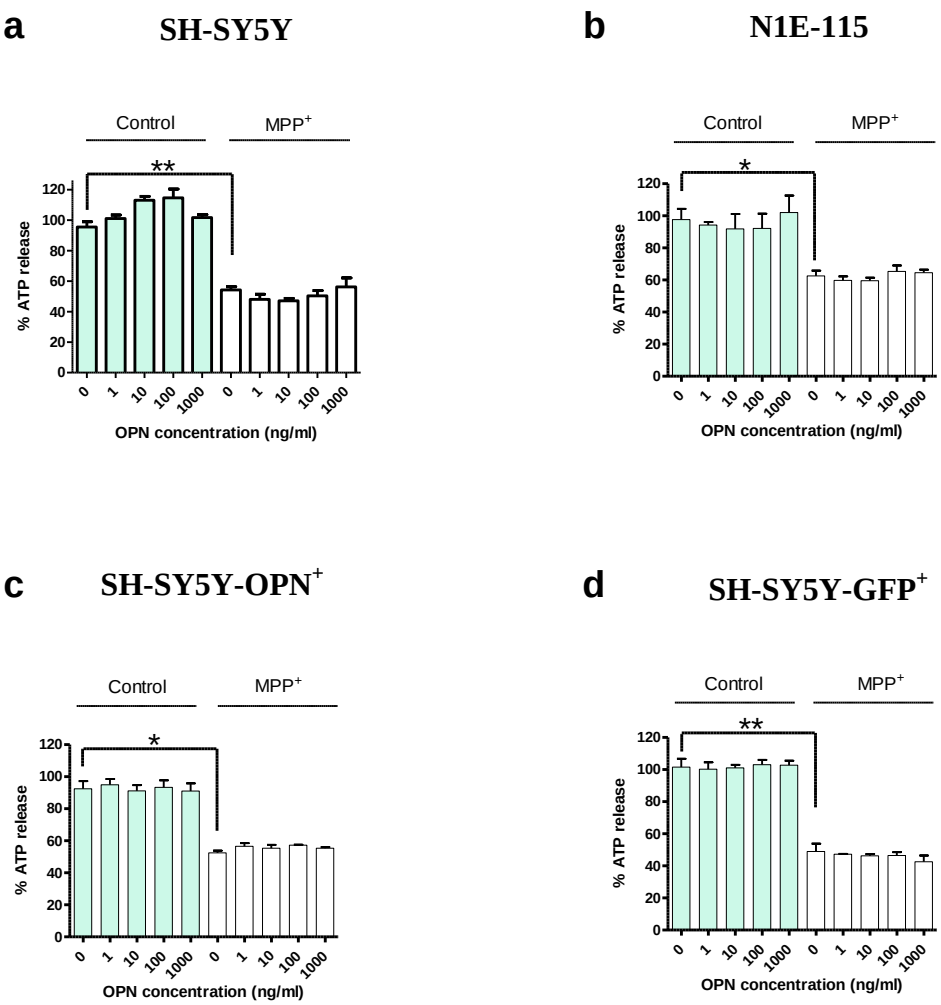

Figure 3.12 The effect of OPN treatment on MPP^+ induced cell death in SH-SY5Y, N1E-115, SH-SY5Y-OPN^+ and SH-SY5Y-GFP^+ cells as measured by ATP assay.

(a-d) Cells were treated with OPN (1-1000ng/ml) 24hr prior to challenge with MPP^+ at appropriate EC_{50} concentrations (Table 3.2). Control untreated cells were taken as 100% ATP release. Data are expressed as mean ±SEM (n=3). * $p<0.01$, ** $p<0.001$ compared to control (two-way ANOVA followed by Newman Keul's test).

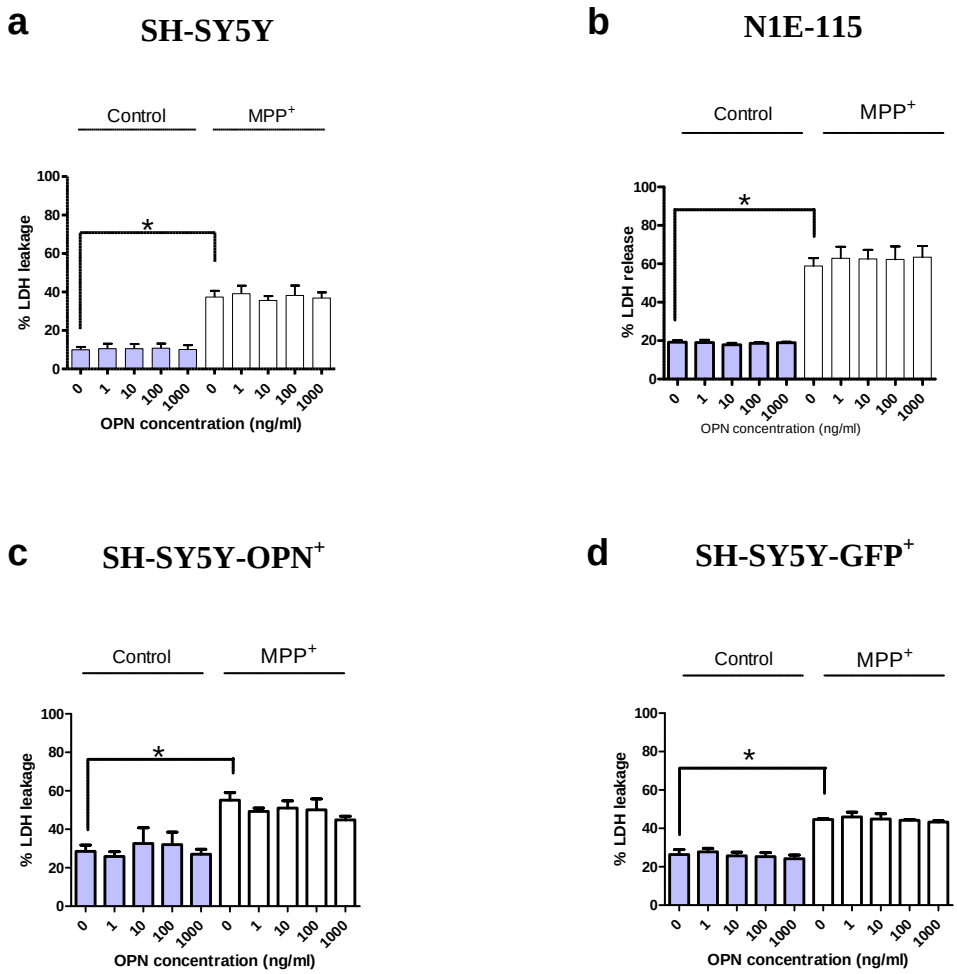

Figure 3.13 The effect of OPN treatment on MPP^+ induced cell death in SH-SY5Y, N1E-115, SH-SY5Y-OPN^+ and SH-SY5Y-GFP^+ cells as measure by LDH assay.

(a-d) Cells were treated with OPN (1-1000ng/ml) 24hr prior to challenge with MPP^+ at appropriate EC_{50} concentrations (Table 3.2). Maximal LDH leakage from lysis buffer treated cells is 100%. Data are expressed as mean ±SEM (n=3). * $p<0.01$ compared to control (two-way ANOVA followed by Newman Keul's test).

3.5.7 The effect of OPN expression on the response to exogenous OPN treatment in H_2O_2 treated cell lines

The effect of OPN expression on the ability of OPN to protect against H_2O_2 toxicity was studied in SH-SY5Y, N1E-115, SH-SY5Y-OPN$^+$ and SH-SY5Y-GFP$^+$ using H_2O_2 at the pre-determined EC_{50} concentrations (Table 3.3). OPN (1-1000ng/ml) treatment alone for 24hrs did not alter LDH leakage or ATP content of cells (Figures 3.14, 3.15). Treatment with H_2O_2 for 24hrs caused significant cell death of about 40-50% as measured by LDH leakage. Pre-treatment of all cells with OPN (1-1000ng/ml) did not induce any changes to toxin–induced cell death (Figure 3.15). This was confirmed by ATP content assay, where treatment with H_2O_2 caused significant cell death of 40-50% but pre-treatment with OPN (1-1000ng/ml) did not confer any protection to all cell lines (Figure 3.14).

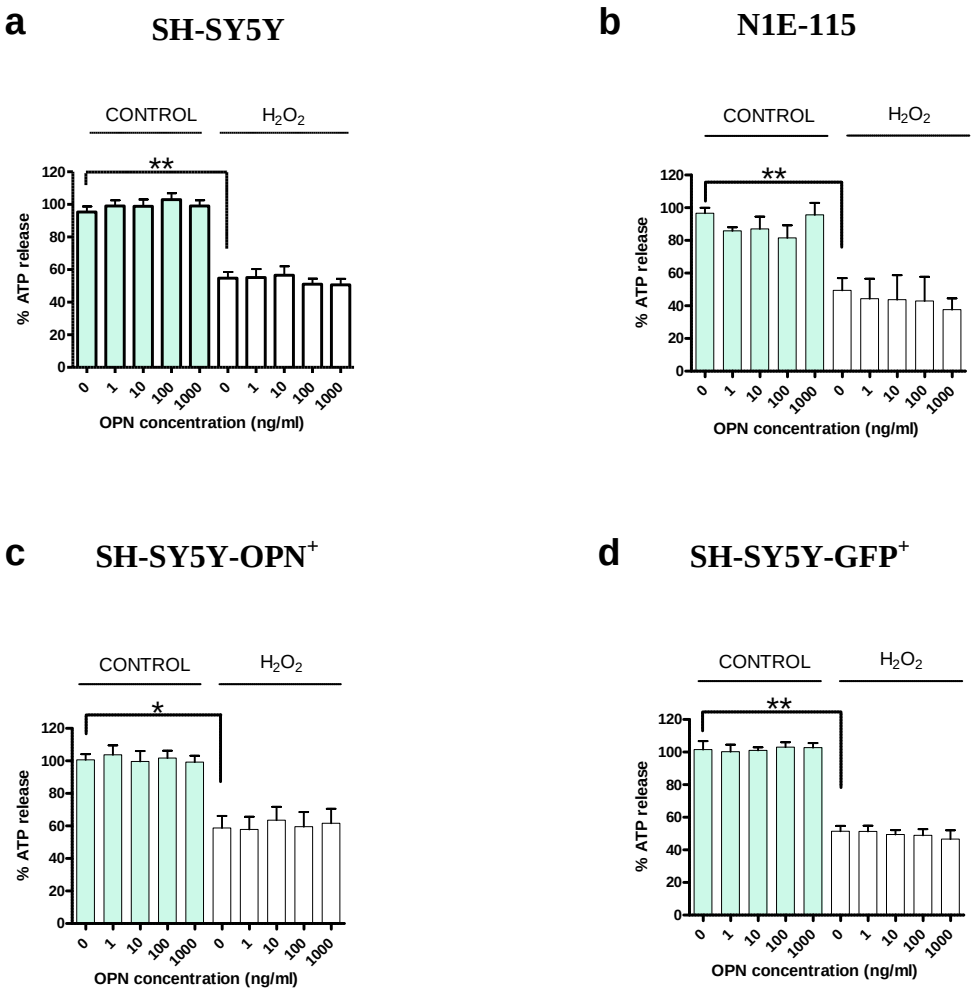

Figure 3.14 The effect of OPN treatment on H_2O_2 induced cell death in SH-SY5Y, N1E-115, SH-SY5Y-OPN$^+$ and SH-SY5Y-GFP$^+$ cells as measured by ATP assay.

(a-d) Cells were treated with OPN (1-1000ng/ml) 24hr prior to challenge with H_2O_2 at the relevant EC_{50} concentrations (Table 3.3). Control untreated cells were taken as 100% ATP release. Data are expressed as mean ±SEM (n=3). *p<0.01, ** p<0.001 compared to control (two-way ANOVA followed by Newman Keul's test).

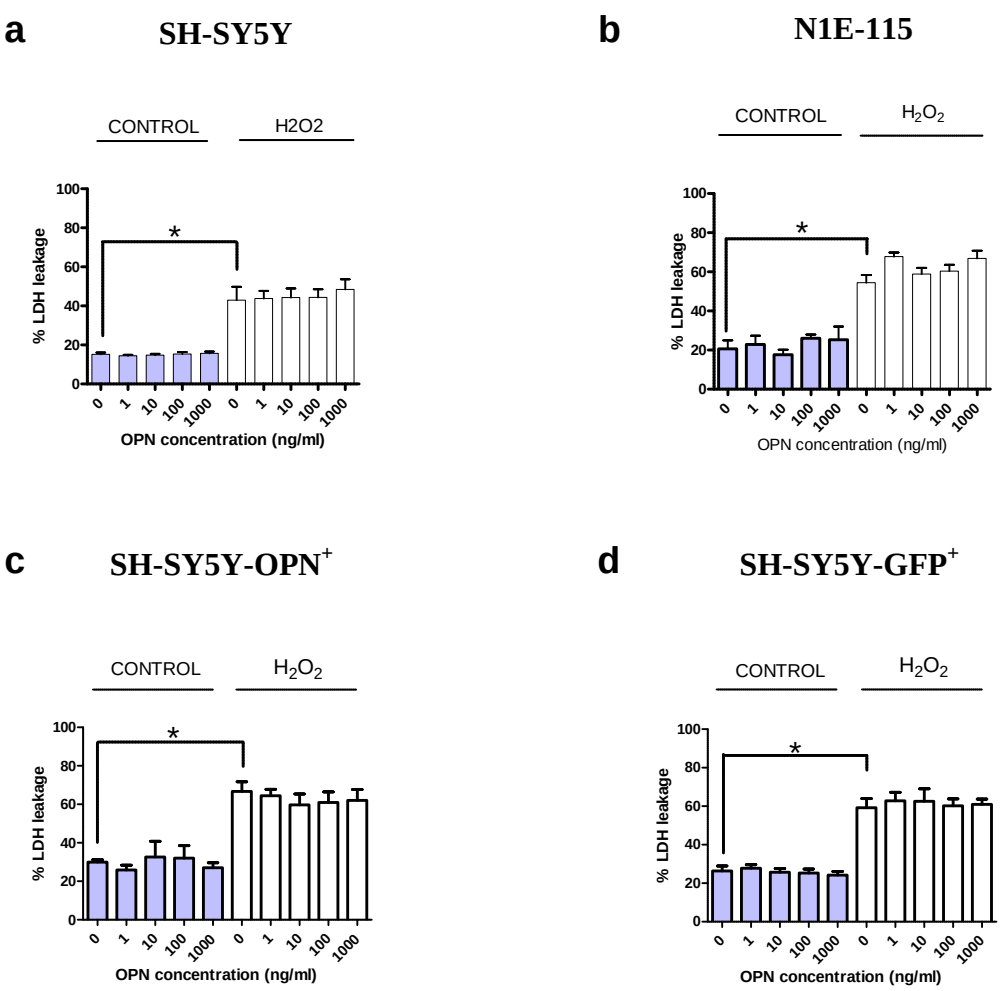

Figure 3.15 The effect of OPN treatment on H_2O_2 induced cell death in SH-SY5Y, N1E-115, SH-SY5Y-OPN$^+$ and SH-SY5Y-GFP$^+$ cells as measured by LDH assay.

(a-d) Cells were treated with OPN (1-1000ng/ml) 24hr prior to challenge with H_2O_2 at the appropriate EC_{50} concentrations (Table 3.3). Maximal LDH leakage from lysis buffer treated cells is 100%. Data are expressed as mean ±SEM (n=3). * $p<0.01$ compared to control (two-way ANOVA followed by Newman Keul's test).

3.4 Discussion

It was hypothesized that endogenous OPN expression confers intrinsic protection to cell lines and that exogenous OPN treatment protects cell lines against toxic insult via binding to Igα_v, Igβ_3, Igβ_1 or CD44 receptors. In order to test this hypothesis, cell lines were characterised for OPN, Igα_v, Igβ_3, Igβ_1 and CD44 expression. Then, the susceptibility of OPN-null and OPN-positive cells to toxin-induced cell death was compared. Finally, the effect of exogenous OPN treatment on toxin-induced cell death in OPN-null and OPN-positive cells was investigated.

3.4.1 Choice of cell lines suitable for addressing the hypothesis

Characterisation of OPN and receptors phenotype

The TH phenotype of the cell lines used was confirmed. SH-SY5Y, N1E-115, SK-NMC and PC-12 cells, but not Ntera-2, expressed TH. This is in agreement with previous reports, where SH-SY5Y, N1E-115, SK-NMC and PC-12 cells but not Ntera-2 were dopaminergic in character (Amano *et al.*, 1972; Markey *et al.*, 1980; Stallcup, 1979).

The presence of OPN protein in N1E-115 and PC-12 cell lines was demonstrated by immunofluorescence and Western blotting techniques whereas SH-SY5Y, SK-NMC and Ntera-2 cells did not express OPN. This is the first time OPN expression has been investigated in these cell lines. On the other hand, α_v and β_3 integrin receptor subunits were expressed by all five cell lines. This is consistent with previous studies where SH-SY5Y, Ntera-2 and PC-12 were reported to express the α_v integrin subunit (Choi *et al.*, 1994; Gibson *et al.*, 2005; Lin *et al.*, 1993) and PC-12 cells were positive for β_3 (Tomaselli *et al.*, 1990). While (Linnala *et al.*, 1997) found no positive immunostaining for α_v and β_3 integrin subunits in undifferentiated SH-SY5Y, (Choi *et al.*, 1994) reported detectable levels of the $\alpha_v\beta_3$ integrin heterodimer in these cells using immunoprecipitation (Choi *et al.*, 1994; Linnala *et al.*, 1997). Importantly, this is the first report of the expression of α_v and β_3 integrins in N1E-115 and SK-NMC cell lines. All cell lines showed positive immunostaining for the β_1 integrin receptor subunit and this is not surprising, as the largest subgroup of integrin heterodimers is formed by β_1 integrin. Indeed, there have been previous reports on the expression of β_1 integrin by all five cell lines

(Amendola *et al.*, 2001; Amy *et al.*, 2004; Cherubini *et al.*, 2005; Chuluyan *et al.*, 2000; Chuluyan *et al.*, 1998; Gibson *et al.*, 2005; Miyashita *et al.*, 2004; Tomaselli *et al.*, 1990; Wang *et al.*, 2006). In addition, all cell lines expressed detectable levels of the CD44 receptor. Weak expression of this receptor was reported in SH-SY5Y cells using reverse transcriptase-polymerase chain reactions and flow cytometry (Gross *et al.*, 1995; Yan *et al.*, 2003). In another report, 11% of SHSY5Y cells expressed CD44H which is the standard or haematopoietic isoform (Gross *et al.*, 1994). In the present study, the antibody used to detect the CD44 receptor recognises all isoforms of this receptor as it is raised against the conserved trans-membrane domain. This antibody was used because it is still not established which CD44 isoforms mediate OPN's pro-survival effects (Chapter 1). The presence of CD44 receptor has not previously been investigated in the other four cell lines before.

In summary, all cell lines expressed $Ig\alpha_v$, $Ig\beta_3$, $Ig\beta_1$ and CD44 and therefore, in terms of receptor expression criterion, they were all suitable for addressing the aims of this Chapter.

Choice of cells

The criteria set for choosing cell lines suitable for addressing the aims of this chapter are explained in Section 3.3.3. In summary, it is preferable that cells chosen are neuronal and dopaminergic in character, cell lines need to express $Ig\alpha_v$, $Ig\beta_3$, $Ig\beta_1$ and CD44 receptors and either express OPN endogenously or be non-OPN expressing. OPN expressing N1E-115 cells were chosen over PC12 cells as the former cells are neuronal while the latter are not. Non-OPN expressing SH-SY5Y cells were chosen over NTera-2 as the latter are not dopaminergic. Both non-OPN expressing SH-SY5Y and SK-NMC cells meet all the criteria and thus are both suitable candidates, but SH-SY5Y cells were preferred as they are more commonly used in PD research and easier to maintain.

Therefore, OPN-expressing N1E-115 and non-OPN expressing SH-SY5Y cells were selected for further tudies as they differ in their OPN expression profile but also meet all the criteria set for addressing the aims of these studies. In summary, both cell lines are: neuronal, dopaminergic and express $Ig\alpha_v$, $Ig\beta_3$, $Ig\beta_1$ and CD44 receptors. SH-SY5Y cells were transfected to express OPN protein (SH-SY5Y-OPN^+) in order to investigate the effect of introducing OPN expression on their

sensitivity to toxic insult and to OPN challenge. *N1E-115, SH-SY5Y, SH-SY5Y-OPN*$^+$ and *SH-SY5Y-GFP*$^+$ were therefore used for further investigation.

3.4.2 Endogenous OPN expression does not affect the sensitivity of cell lines to toxin-induced cell death

MPP^+ and H_2O_2 have been reported to induce apoptotic cell death in cell lines at concentrations similar to those used in the present study: MPP^+ (1.5-3 mM), H_2O_2 (361-417µM) (Dennis *et al.*, 2003; Fall *et al.*, 1999; Gomez *et al.*, 2001; King *et al.*, 2005; Kitamura *et al.*, 2002; Sheehan *et al.*, 1997; Yang *et al.*, 2006).

MPP^+

As expected, MPP^+ induced concentration dependent cell death in SH-SY5Y, N1E-115, SH-SY5Y-OPN^+ and SH-SY5Y-GFP^+ cell lines. MPP^+ (at the highest concentration; 10mM) did not induce maximal LDH leakage in any of the cell lines, although at this concentration the ATP assay showed total cell death. This reflects the fact that maximal LDH leakage in the assay procedure is defined by the effect of adding a lysis buffer which induces complete necrosis and release of total LDH content of the cells. So, MPP^+ exposure may have initiated apoptosis in all cells but not necrosis and therefore LDH release was not complete.

There was no difference in the potency of MPP^+ to induce cell death in OPN expressing N1E-115 cells or non-OPN expressing SH-SY5Y cells when measured by ATP or LDH assays. By contrast, MPP^+ was less potent in inducing cell death in SH-SY5Y-OPN^+ than in SH-SY5Y and N1E-115 cells. However, MPP^+ was also less potent in inducing cell death in control transfected SH-SY5Y-GFP^+ cells, indicating that this is an effect of the transfection process. In conclusion, endogenous OPN expression did not affect the sensitivity of cells to MPP^+ toxicity.

H_2O_2

Again, as expected, H_2O_2 induced a concentration dependent cell death in SH-SY5Y, N1E-115, SH-SY5Y-OPN^+ and SH-SY5Y-GFP^+ cell lines. It also did not cause maximal LDH leakage and this may be due to the same reasons outlined for MPP^+. At the highest concentration used (800µM), H_2O_2 caused more cell death in OPN transfected and control transfected cells compared to non-transfected SH-SY5Y and N1E-115 cells, suggesting that again this is an effect of the transfection process.

H_2O_2 was more potent in inducing cell death in N1E-115 cells than in SH-SY5Y cells suggesting that OPN expression may render cells more vulnerable to H_2O_2 toxicity, as measured by the ATP assay. However, the potency of H_2O_2 in SH-SY5Y-OPN$^+$ cells was not different to that in SH-SY5Y-GFP$^+$ suggesting that this does not reflect OPN expression but rather the nature of the cells. Unlike the ATP assay, the LDH assay showed no difference between cells in the potency of H_2O_2. The ATP assay may be more sensitive to the pro-oxidative effects of H_2O_2 as the ATP content of cells may be affected by the effect of oxidative stress on mitochondria.

In summary, endogenous OPN expression in N1E-115 cells and the introduction of OPN expression in SH-SY5Y-OPN$^+$ cells did not alter their sensitivity to toxin induced cell death. Therefore, endogenous OPN expression does not confer protection against toxic insult. Although OPN transfection did not seem to be protective in this study, it is noteworthy that, in both assays potencies of MPP$^+$ or H_2O_2 in SH-SY5Y- OPN$^+$ are not different to those in SH-SY5Y-GFP$^+$ control. This shows that transfection with OPN does not confer a higher vulnerability to cells, suggesting that introducing expression of this protein in cells is not in itself harmful.

3.4.3 Exogenous OPN treatment does not protect cells from toxin induced cell death

Exogenous OPN treatment alone did not alter cell viability in any of the cell lines. As expected, MPP$^+$ and H_2O_2 induced a significant increase in death in all cell lines. However, exogenous OPN treatment 24hrs prior to toxic challenge did not protect any cell line from cell death as measured by either ATP or LDH assays. This shows for the first time that exogenous OPN treatment does not protect cell lines against toxic insult regardless of the OPN expression phenotype. In addition, OPN did not protect cells although they expressed Igα_v, Igβ_3, Igβ_1 and CD44 receptors which have been previously shown to mediate protective actions of OPN (Caers *et al.*, 2006; Lee *et al.*, 2007; Lin *et al.*, 2001; Scatena *et al.*, 1998). This could be due to a number of reasons. First, pro-survival actions of OPN may not be mediated through Igα_v, Igβ_3, Igβ_1 and CD44 receptors in these cell lines. In fact, none of the integrin receptor subunits bind to OPN by themselves but need to form dimeric complexes for a functional receptor unit to be present and to bind OPN (Chapter 1).

For instance, Igβ_1 associates not only with α_v but with four other α receptor subunits (α_5, α_8, α_4 and α_9), one or more of these may be necessary for the protective effects of OPN but may not be present in the cell lines. In addition, the antibody used to detect CD44 receptors in this study detects all CD44 isoforms as it binds to the conserved trans-membrane domain. There are differing reports as to which CD44 isoform OPN binds (Chapter 1). Since it is not known which isoforms of CD44 are expressed in these cell lines, they may well be lacking an isoform essential for mediating the protective effects of OPN. Importantly, interaction of OPN with different receptors may have different functional consequences (Katagiri *et al.*, 1999; Liaw *et al.*, 1995; Yue *et al.*, 1994). This suggests that OPN may require a different mechanism through different receptors that may not be present in these cell lines.

Second, recombinant OPN, added exogenously to cell lines, is not phosphorylated (Ashkar *et al.*, 1993b) and can undergo autophosphorylation on tyrosine residues *in-vitro* in the presence of ATP or GTP (Ashkar *et al.*, 1993a). Endogenous OPN on the other hand, has 58 consensus potential phosphorylation sites for various protein kinases and depending on tissue type, it is phosphorylated on many sites (Ashkar *et al.*, 1993b). *In-vivo*, some phosphorylation is indeed extracellular (Krane *et al.*, 1962) suggesting that recombinant OPN exogenously added to cultures could be phosphorylated. However, it was beyond the scope of this study to determine the phosphorylation state of OPN expressed by these cell lines or the *in-vitro* phosphorylation of exogenous OPN. The phosphorylation state of OPN is important as it may be crucial to its function by determining whether it interacts with cells or the ECM (Ek-Rylander *et al.*, 1994; Nemir *et al.*, 1989) and by enhancing its binding to integrin receptors via the RGD domain. In fact, the GRGDS integrin binding sequence in OPN is located only 3 residues away from tyr150, a highly conserved residue in mammalian OPN. Tyr150 is also flanked by the thrombin cleavage sites and this is a potential site for autophosphorylation, that may alter binding to integrin receptors. Phosphorylation of Ser147; located within the GRGDS integrin binding sequence, may also regulate the interaction between OPN and receptors on cells (Denhardt *et al.*, 1995). Moreover, phosphorylation of OPN varies in different tissues, for instance milk OPN versus bone OPN (Keykhosravani *et al.*, 2005), indicating that phosphorylation may regulate OPN's function. OPN secreted from JB6 mouse cells is non-phosphorylated, but that secreted from

tumorigenic JB6 cells is phosphorylated (Chang *et al.*, 1991), suggesting that phosphorylation of the protein is also dependent on the physiological state of the cell. Therefore, phosphorylation of endogenous OPN in N1E-115 and SH-SY5Y neuroblastoma cell lines may be different to that in other cell types and this may explain lack of protection. The phosphorylation state of OPN expressed by transfected SH-SY5Y-OPN^{+} is not known and may also be the reason why no protection to the cells was seen.

Third, OPN is subject to proteolytic cleavage by thrombin (Senger *et al.*, 1989) and MMPs (Agnihotri *et al.*, 2001), which may regulate its function. Cleavage by both thrombin and MMPs occurs at close proximity to the integrin recognition sequence RGD (Agnihotri *et al.*, 2001; Smith *et al.*, 1996; Yokasaki *et al.*, 2000). In fact, proteolytic cleavage may alter the binding affinity to integrin receptors as one study showed that cellular adhesion to full length OPN mediated via $\alpha_5\beta_1$ and $\alpha_9\beta_1$ was minimal and while thrombin cleavage enhanced adhesion, cleavage by MMPs inhibited it (Yokosaki *et al.*, 2005). In addition, thrombin cleaved OPN induces greater cell attachment than the full length protein (Senger *et al.*, 1994), suggesting that cleavage is involved in regulating OPN actions. In certain cells such as melanoma cells, cell binding occurs only with the thrombin cleaved OPN (Smith *et al.*, 1998). On the other hand, OPN full length had a greater anti-apoptotic effect on neutrophils than the thrombin cleaved protein (Sharif *et al.*, 2009). Furthermore, a recent study showed for the first time that OPN is also cleaved by caspase-8 and that mutating OPN to be resistant to this cleavage renders it more efficient in suppressing hypoxia/regeneration induced cell death (Kim *et al.*, 2009). Therefore, the processing of OPN in the neuronal cell lines environment may be different to that in other environments where it was previously shown to be protective.

In summary, neuronal cell lines used in this study may not have the required receptors to mediate pro-survival actions of OPN, and/or OPN may not be modified by these cell lines to function as a protective molecule. OPN is found in many tissues serving diverse functions and it is possible that post-translational modifications of OPN are different in different cell types. Post-translational modifications of OPN were different in proliferating versus mineralizing osteoblasts (Kasugai *et al.*, 1991; Kubota *et al.*, 1989) and normal versus transformed kidney cells (Nemir *et al.*, 1989). OPN produced by tumor cells seemed to be functionally

different compared to OPN produced by host macrophages (Crawford *et al.*, 1998). These diverse functions may also be explained by a different receptor repertoire in different cell types.

Additionally, the cell line model provides a continuous supply of 'mono-typic' neuronal cells with dopaminergic characteristics but they lack the complexity of the real picture, where there are different cell types most notably glial cells which release neurotrophic factors that support neuronal function. OPN has anti-inflammatory actions and it is involved in tissue remodelling and regeneration (Denhardt *et al.*, 1993; Scatena *et al.*, 2007; Weber *et al.*, 1996b) and hence may need more physiologically relevant systems to exert its protective actions. Therefore, primary culture and *in-vivo* models will be used in further studies to investigate the potential neuroprotective effects of OPN.

3.4.4 Conclusion

In conclusion, these data confirm the toxic effect of MPP^+ and H_2O_2 in SH-SY5Y and N1E-115 cell lines. They also showed, for the first time, that endogenous OPN expression does not confer altered vulnerability to toxins and that exogenous OPN treatment does not protect cell lines against toxic insult. This makes the situation difficult as we started from the hypothesis that differential OPN expression in cell lines may have different effects but this was not the case and even exogenous OPN addition was not protective in the cell lines. Therefore, the next step should be to use more biologically relevant systems.

Chapter 4 OPN prevents toxin induced dopaminergic cell death in VM cultures through an integrin receptor interaction.

4.1 Introduction

In the previous chapter, OPN did not protect dopaminergic neuronal cell lines against toxin-induced cell death despite the presence of Igα_v, Igβ_3, Igβ_1 and CD44 receptors. This was surprising as it was previously shown that OPN has properties of a potential protective agent mediated through these receptors (Chapter1). In addition, a study was published as this work was progressing showing the ability of an RGD containing peptide fragment of OPN to inhibit toxin induced cell death in primary VM culture (Iczkiewicz *et al.*, 2010). There are a number of reasons why this might occur as discussed in Chapter 3. In brief, post-translational modifications affecting OPN function are different in various cell types and processing of OPN protein in the cell lines used in those studies may not support a pro-survival function. Also, the pro-survival effects of OPN may be mediated via receptors, other than Igα_v, Igβ_3, Igβ_1 and CD44, which are not expressed in the cell lines used. However, in light of the newly published findings of neuroprotective effects of an RGD containing fragment of OPN in primary culture (Iczkiewicz *et al.*, 2010), there may be other potential reasons for this apparent discrepancy.

First, the fragment found to be protective was based on the concept that the RGD binding domain was essential for activity through an action on integrin receptors whereas the intact protein is associated with multiple potential neuroprotective effects and these may not require the integrin interaction or may require additional interactions. It is possible that such alternative mechanisms may not be present in cell lines but can be found in primary VM culture. Second, the cleavage of OPN at the thrombin cleavage site immediately adjacent to portion of the molecule that interacts with integrin receptors enhances its binding to integrin receptors (Smith *et al.*, 1998). Thus the full length protein may not possess the protective effects previously seen with a fragment, but this has never been tested in PD models. In fact, the concept that the OPN fragment acts through an integrin mechanism is purely based on association, and the presence of these receptors in primary VM culture has never been investigated. In addition, no previous study has investigated whether integrin receptor antagonists can prevent the protective effect of OPN in VM culture.

Another important difference between cell lines and primary VM culture is the presence of glial cells. This may be extremely important since a key activity of OPN is in the regulation of inflammatory processes (Chapter 1). As a consequence, OPN may affect the response of glial cells to toxic insults, but whether OPN alters glial cell activation or number in VM cultures has not been previously investigated. Based on studies on the effects of OPN on inflammatory cells in the periphery, a direct effect through integrin or CD44 receptors might be anticipated. But again no attempt has been made to look at this in relation to VM cultures or PD. It is also not known whether inflammatory or dopaminergic cells in the VM culture express these receptors.

4.1.1 Hypothesis

It was hypothesized that OPN protects dopaminergic neurones of the primary VM culture against toxic insult and that this effect is mediated via an effect on glial cells through binding to integrin and/or CD44 receptors.

4.1.2 Aims

In order to test the hypothesis, studies were performed with the following aims:

- To characterise the primary VM culture for dopaminergic and GABA-ergic neurones and glial cells.
- To characterise the primary VM culture for the expression of OPN, Igα_v, Igβ_3, Igβ_1 and CD44.
- To investigate the effect of exogenous OPN treatment on MPP^+ toxicity in VM culture.
- To investigate the role of integrin and CD44 receptors in the protective actions of OPN.
- To investigate the involvement of glial cells in the protective actions of OPN.

4.2 Methods

The embryonic ventral mesencephalon develops into the SN and the ventral tegmental area (VTA) in the adult rat. When E14 VM tissue is cultured *in-vitro*, the cells differentiate into dopaminergic and GABA-ergic neurones and glial cells (Gilbert *et al.*, 2003; Rodriguez-Pallares *et al.*, 2008; Shimoda *et al.*, 1992). Specific dopaminergic toxins can be used to target dopaminergic cells in this culture, producing an *in-vitro* model of dopaminergic cell death (McNaught *et al.*, 1999b; Rodriguez-Pallares *et al.*, 2008). This model was used to study neuroprotective effects of OPN against MPP^+ toxicity and investigate the role of integrins and CD44 and glial cells in the neuroprotective effects. Details of methods used are described below.

4.2.1 Dissection of the ventral mesencephalon

Ventral mesencephalons were dissected from foetuses from E14 pregnant Wistar rats as described in section 2.3.1. Briefly, foetuses were dissected under a dissection microscope (10x; Vickers Instruments, UK) by making a cut from the mouth to the VM flexure. This was followed by two sagittal cuts to separate out the mesencephalic region, one anterior and one posterior to the peak of the flexure. The ventral part of the mesencephalic segment was obtained by cutting caudally down each side of the ventricle.

4.2.2 Preparation of primary VM cultures

Primary VM cultures were prepared as described in detail in Section 2.3.2. Briefly, cells were plated at a density of $2x10^5$cell/well on poly-D-lysine coated coverslips in 24 well plates (0.5ml full growth medium/well) and grown in a cell culture incubator (Sanyo, 37˚C, 5%CO_2, 100% humidity) until use.

4.2.3 Characterisation of primary VM cultures

Primary VM culture cells were characterised for the dopaminergic cell marker TH, a marker of GABA-ergic neurones; glutamate decarboxylase (GAD), a marker of microglia (OX-42) and a marker of astrocytes (GFAP). At DIV 3, 4 or 5, medium was removed and cells washed with cold DPBS then fixed with cold PFA (4% in PBS) for 15min. Following fixation, cells were stained using immunoperoxidase

cytochemistry for TH and GAD (Section 4.2.4), and counter-stained with Nissl stain as decribed in Section 4.2.4.

4.2.4 Peroxidase immunocytochemistry in VM cultures

Peroxidase immunocytochemistry was carried out in 24 well plates (0.5ml/well) as described in detail in Section 2.3.3. Briefly, following incubation with 20% blocking solution (20% goat serum, 0.05% Triton-X 100 in PBS) for 1hr at room temperature, cells were incubated with primary antibodies (Table 2.3) in 1% blocking solution (1% goat serum in PBS) overnight at room temperature. After three washes with PBS, cells were incubated with appropriate biotinylated secondary antibodies (Table 2.4) diluted in 1% blocking solution for one hour. After incubation with avidin: biotinylated enzyme complex (vectastatin ABC Kit, Vector) colour was visualised with DAB and in characterisation experiments, cells were counterstained with Nissl stain (Section 2.3.3).

4.2.5 Determination of the EC_{50} concentration of MPP^+ in VM cultures

VM cultures were grown on poly-D-lysine coated coverslips in 24 well plates as described in Section 2.3.2. At DIV3, medium was changed to serum free medium (DMEM-Glutamax supplemented with 1% PSN; 500µl). At DIV 4, an aliquot of medium (50µl) was removed from each well and 50µl of MPP^+ (final concentrations: 10-800µM) or vehicle (serum free medium) were added to wells. At DIV5, medium was removed and cells washed three times in cold DPBS (0.5ml/well) then fixed in cold PFA (4%/ 0.1M PBS, 0.5ml/well) for 15min. Cells treated with serum-free media only served as control. Cells were then stained for TH expression as described in Section 4.2.4 and the number of TH positive cells counted as described in Section 4.2.10.

4.2.6 Neuroprotection Assays in VM cultures

VM culture cells were grown on poly-D-lysine coated coverslips in 24 well plates as described in Section 2.3.2. At DIV 3, medium was changed to serum free medium. In order to determine whether OPN exerted neuroprotective effects, the cells were pre-treated with recombinant rat OPN (custom made, GenScript, USA; 1-100ng/ml) diluted in serum free medium three days after plating (DIV 3) by adding an aliquot (50µl) of stock solutions (10 times the desired concentration) to each well

(450µl). After a further 24hr (DIV 4), cells were treated with MPP^+ at the pre-determined EC_{50} concentrations as described in Section 4.2.5. Control cultures were treated with either 1. Serum free only, 2. OPN only or 3. MPP^+ only. Twenty four hours after toxin insult (DIV 5), medium was removed and cells washed three times in cold DPBS (0.5ml/well) then fixed in cold PFA (4% in PBS; 0.5ml/well) for 15min. Cells were then stained for TH expression as described in Section 4.2.4 and the number of remaining TH positive cells counted as described in Section 4.2.10.

4.2.7 Neuroprotection assays using a 15-mer fragment of OPN

In order to investigate whether a small peptide sequence of OPN comprising the integrin binding domain RGD was responsible for the protective effect of OPN, a 15-mer peptide sequence of OPN comprising the integrin-binding domain RGD (TVDVPDGRGDSLAYG) was custom-synthesised (Sigma-Aldrich, UK). Neuroprotection assays were performed as described in Section 4.2.6 but using OPN fragment (1-100ng/ml) instead of full length OPN.

4.2.8 Neuroprotection assays in the presence of integrin inhibitors

Neuroprotection assays were performed as described in Section 4.2.6 with the following modifications. At DIV 3, 30min before adding OPN, non-selective integrin inhibitors (RGDS or GRGDSPK; Sigma-Aldrich, UK) shown previously to inhibit binding to integrin receptors (Dekkers *et al.*, 2010; Onodera *et al.*, 2005) were added at a final concentration range of 1-100nM. OPN was then added and 24hr later, cells were treated with MPP^+ as described in Section 4.2.5. Control cultures were treated with either 1. Serum free only, 2. integrin inhibitor only or 3. MPP^+ only. Cells were fixed at DIV 5, stained for TH expression (Section 4.2.4) and the number of remaining TH positive cells counted as described in Section 4.2.10.

4.2.9 Double labelling fluorescence immunocytochemistry in VM cultures

Double labelling fluorescence immunocytochemistry was employed to identify the cellular localisation $Ig\alpha_v$, $Ig\beta_3$, $Ig\beta_1$ and CD44 receptors in VM cultures. The cellss were stained for $Ig\alpha_v$, $Ig\beta_3$, $Ig\beta_1$ or CD44 and a marker of either dopaminergic neurons (TH), astrocytes (GFAP), microglia (OX-42) or macrophages (ED-1). This technique was carried out in 24 well plates (0.5ml/well) as described in Section 2.3.5.

Briefly, after incubation with 20% blocking solution (20% goat serum, 0.1% Triton-X 100 in PBS) for one hour, the cells were incubated with a mixture of two primary antibodies (Table 2.3) for detecting the two desired antigens, diluted in a 1% blocking solution (1% goat serum, 0.1% Triton-X 100 in PBS) overnight at 4˚C. On the next day cells were incubated with the relevant secondary antibodies (Table 2.4) conjugated with Alexa Fluor 594 or Alexa Fluor 488 for one hour in a darkened environment. A Zeiss Axioskop microscope was used to examine the sections.

4.2.10 Data and statistical analysis

Immunoreactive cells were counted at x10 magnification in ten randomly selected areas per coverslip (total area=10mm^2), with three to four coverslips per experiment as described in Section 2.3.4. For characterisation of VM cultures, the number of TH positive or GAD positive cells were expressed as percentage of the total number of cresyl violet stained cells (Section 2.3.4). Non-linear regression curve was fitted to log concentration-response data and EC_{50} concentration of MPP^+ calculated.

Data are expressed as mean±SEM of three to four individual experiments (n=3-4). One-way ANOVA followed by Newman Keuls post hoc test was performed on MPP^+ concentration-response data. Data from neuroprotection studies were analysed by two-way ANOVA followed by Newman Keuls' test. $P<0.05$ was considered significant. GraphPad Prism 5 was used to analyse the Data.

4.3 Results

4.3.1 Characterisation of the VM culture

VM cultures were characterised at DIV 3, 4 and 5 for the proportion of dopaminergic and GABAergic neurones by immunocytochemistry for TH and GAD respectively and compared to the total number of cresyl violet stained cells. The composition of cultures was the same in all days: at DIV 3, 6% TH immunopositive cells and 51% GAD immunopositive, at DIV 4, 5% TH immunopositive cells and 52% GAD immunopositive cells and at DIV 5, 5% TH immunopositive cells and 53% GAD immunopositive cells (Figure 4.1). TH and GAD staining was observed on cell bodies as well as processes.

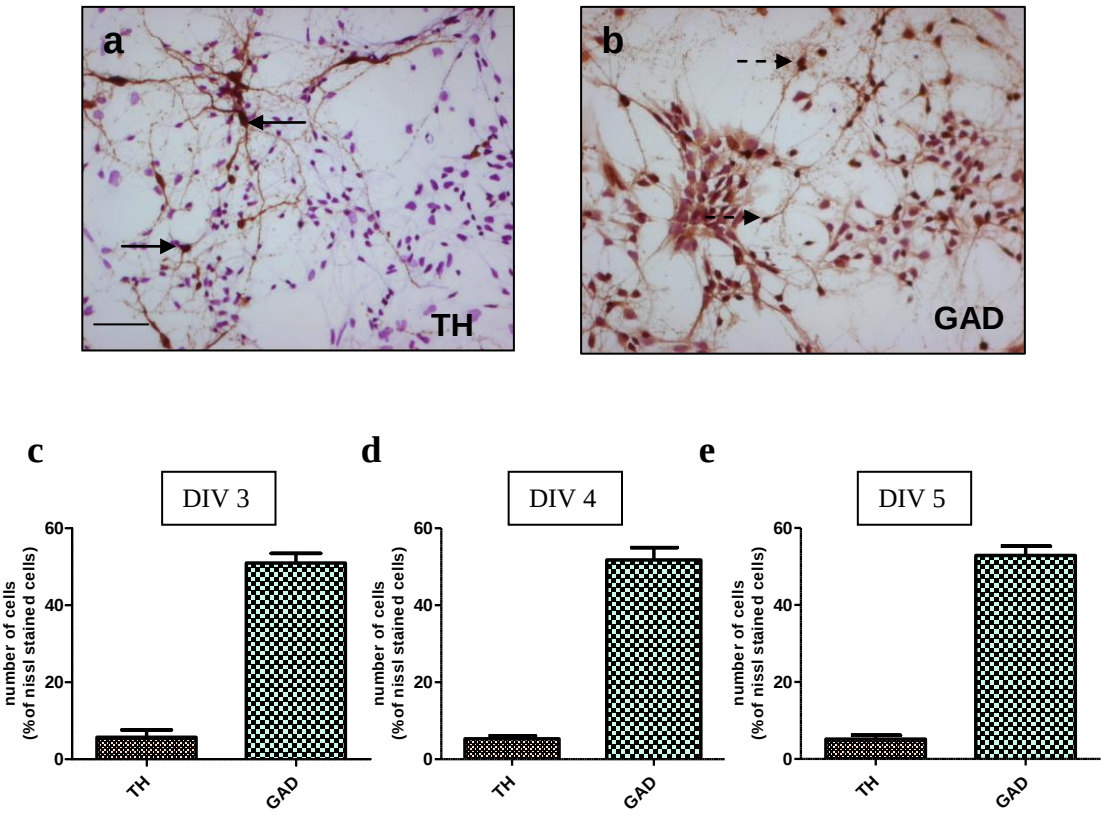

Figure 4.1 Characterisation of the VM culture.

(a-b) Representative photomicrographs of VM cultures from DIV 3, 4 and 5 stained by peroxidase immunocytochemistry for TH (a) or GAD (b) and counter-stained with cresyl violet. ⟶ TH stained cell bodies, - - ▸ GAD stained cell bodies. Magnification 20x, scale bar = 200μm and is representative of both images. (c- e) TH positive and GAD positive cells were counted in cultures fixed at DIV 3 (c), DIV 4 (d) or DIV 5 (e). Data are expressed as % of cresyl violet stained cells, mean ±SEM (n=3).

4.3.1.a Investigation of OPN and Igα$_v$, Igβ$_3$, Igβ$_1$ and CD44 receptors expression in dopaminergic neurones of the VM culture

The expression of OPN, Igα$_v$, Igβ$_3$, Igβ$_1$ and CD44 receptors was determined in dopaminergic cells of the VM culture. VM cultures were fixed at DIV 3 or 5 and stained for TH, OPN, Igα$_v$, Igβ$_3$, Igβ$_1$ or CD44 using double-labelling immunofluorescence. OPN and Igα$_v$, Igβ$_3$, Igβ$_1$ and CD44 receptors were all expressed in VM culture cells (Figures 4.2, 4.3). OPN staining appeared in the cell body, mainly in the cytoplasm and also on processes (Figure 4.2). Igα$_v$, Igβ$_3$ and Igβ$_1$ immunoreactivity appeared strongly in cell nuclei with fainter staining on the cytoplasm and few processes (Figure 4.3). CD44 receptor immunoreactivity appeared in the whole cell body with fainter staining on processes (Figure 4.3).

Double immunofluorescence labelling with anti-TH antibody showed that TH immunopositive cells expressed OPN (Figure 4.2) but also Igα$_v$, Igβ$_3$, Igβ$_1$ and CD44 receptors thought to mediate its pro-survival actions (Figure 4.3). OPN and its receptors were also expressed on other cells that were not TH immunopositive (Figures 4.2, 4.3).

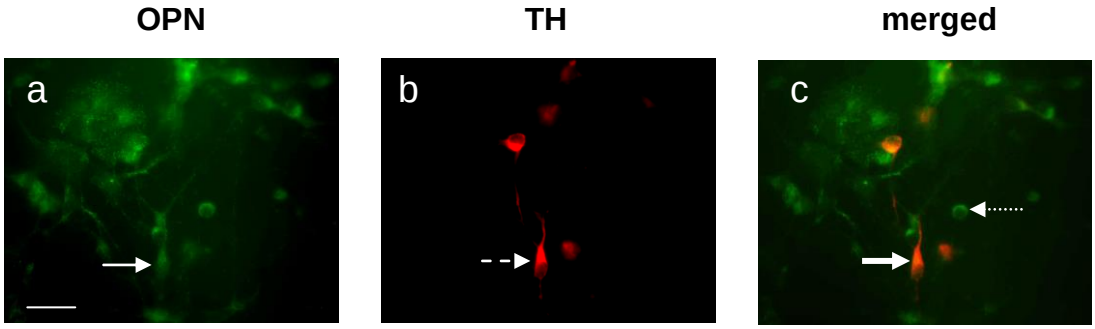

Figure 4.2 OPN expression in TH immunopositive cells in VM cultures.

Representative photomicrographs showing Immunofluorescence staining of TH (red), OPN (green) in VM culture cells. ⟶ OPN staining, - - ▸ TH staining, ➡ co-localisation of OPN and TH, ⋯▸ OPN positive cell which is not TH positive. Magnification 20x, Scale bar = 50μm and is representative of all images.

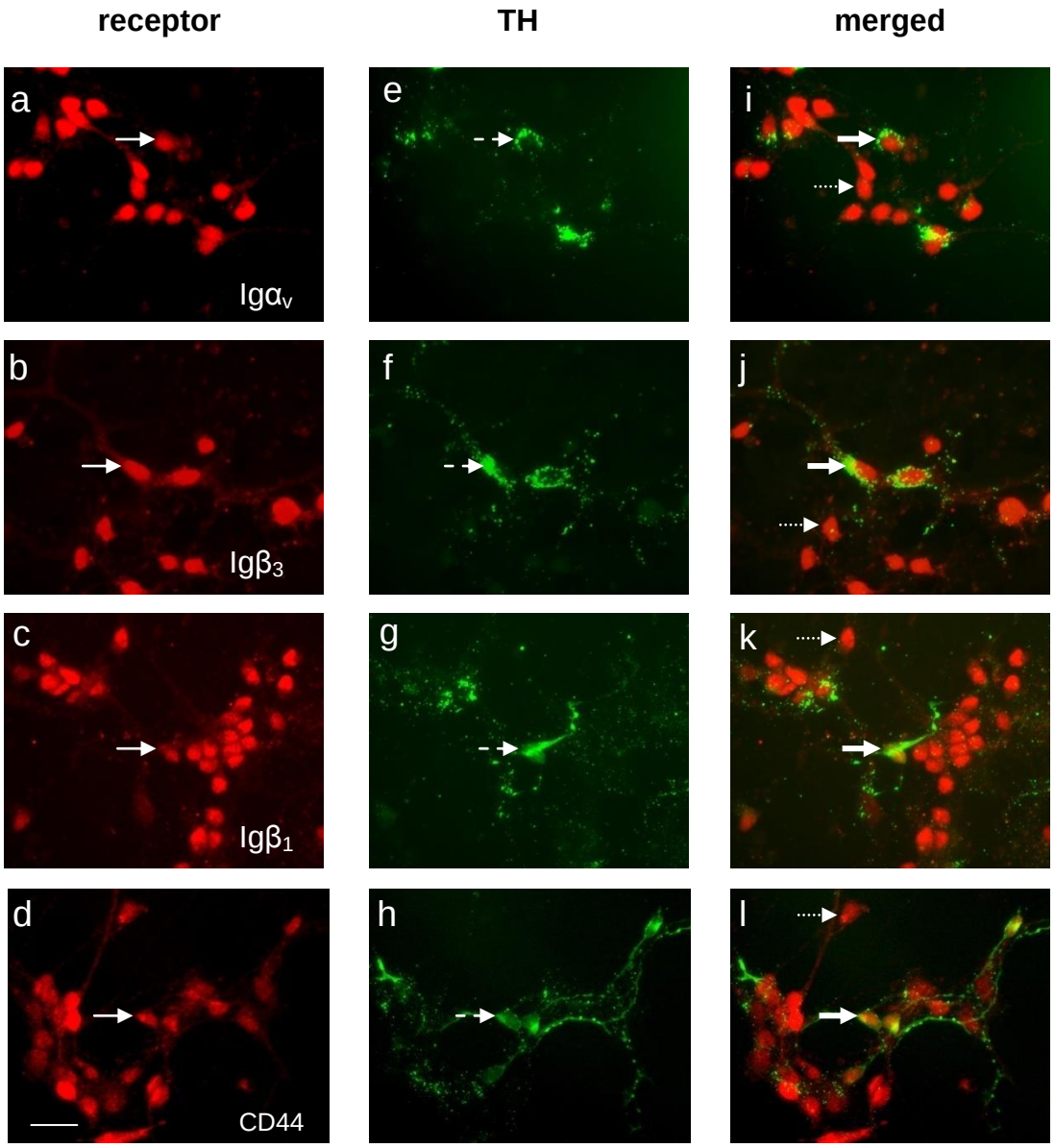

Figure 4.3 Expression of Igα$_v$, Igβ$_3$, Igβ$_1$ and CD44 receptors in TH immuopositive cells in VM cultures.

Representative photomicrographs showing Igα$_v$, Igβ$_3$, Igβ$_1$ or CD44 receptor positive cells (red; a-d; →) TH positive cells (green; e-h; - →), cells expressing both TH and receptor (i-l; ➡), cells expressing receptor but not TH (i-l; ⋯►) in VM cultures. Magnification x40, Scale bar = 10μm and is representative of all images.

4.3.1.b Investigation of glial cell presence in the VM culture

In order to determine the difference in cell composition between cell lines and primary VM culture, presence of glial cells in the VM culture was investigated at DIV 3. Immunoperoxidase staining for glial cell markers showed presence of OX-42 and GFAP positive cells (Figure 4.4). OX-42 immunoreactivity appeared on round cells with no processes while GFAP positive cells had few processes (Figure 4.4).

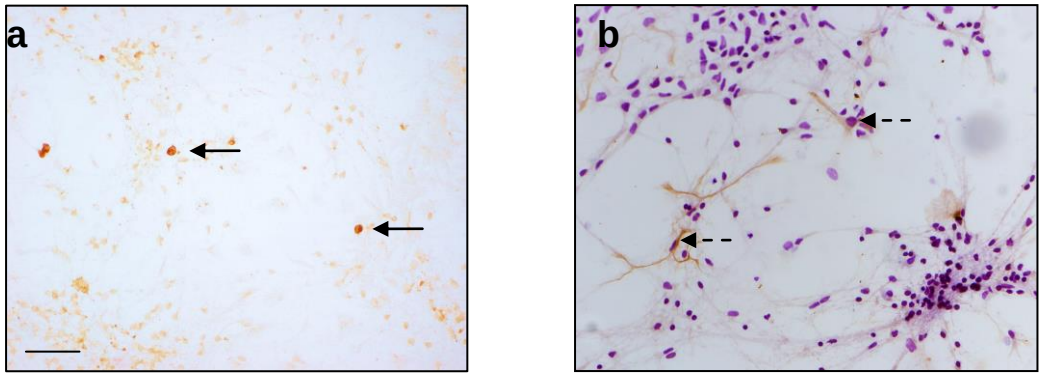

Figure 4.4 Expression of glial cell markers in VM cultures.

Representative photomicrographs showing (a) OX-42 immunoreactivity (⟶), (b) GFAP immunoreactivity (brown, - - ▶) in VM cultures, cells were counterstained with cresyl violet (blue). Magnification x20, scale bar= 200µm and is representative of both images.

4.3.1.c The expression of Igα_v, Igβ_3, Igβ_1 and CD44 receptors in inflammatory cells in VM cultures

The expression of Igα_v, Igβ_3, Igβ_1 and CD44 receptors was investigated in inflammatory cells in the VM culture. VM cultures were fixed at DIV 3 or 5 and immunostained for OX-42, GFAP or ED-1 and Igα_v, Igβ_3, Igβ_1 or CD44 using double-labelling immunofluorescence. Double immunofluorescence showed that OX-42 positive microglia, GFAP positive astrocytes and ED-1 positive macrophages expressed Igα_v, Igβ_3, Igβ_1 and CD44 receptors, thought to mediate OPN's pro-survival actions (Figures 4.5-4.7). Igα_v, Igβ_3, Igβ_1 and CD44 receptors were also expressed on other cells that were not immunopositive for the inflammatory marker investigated (Figures 4.5-4.7). In microglia, astrocytes and macrophages, Igα_v, Igβ_3 and Igβ_1 staining was localised to cell bodies and more intense immunoreactivity was observed in nuclei (Figures 4.5-4.7). Integrin staining was not clearly seen on the processes of astrocytes. CD44 staining was observed on cell bodies of microglia, astrocytes and macrophages and also appeared on astrocytes processes (Figures 4.5-4.7).

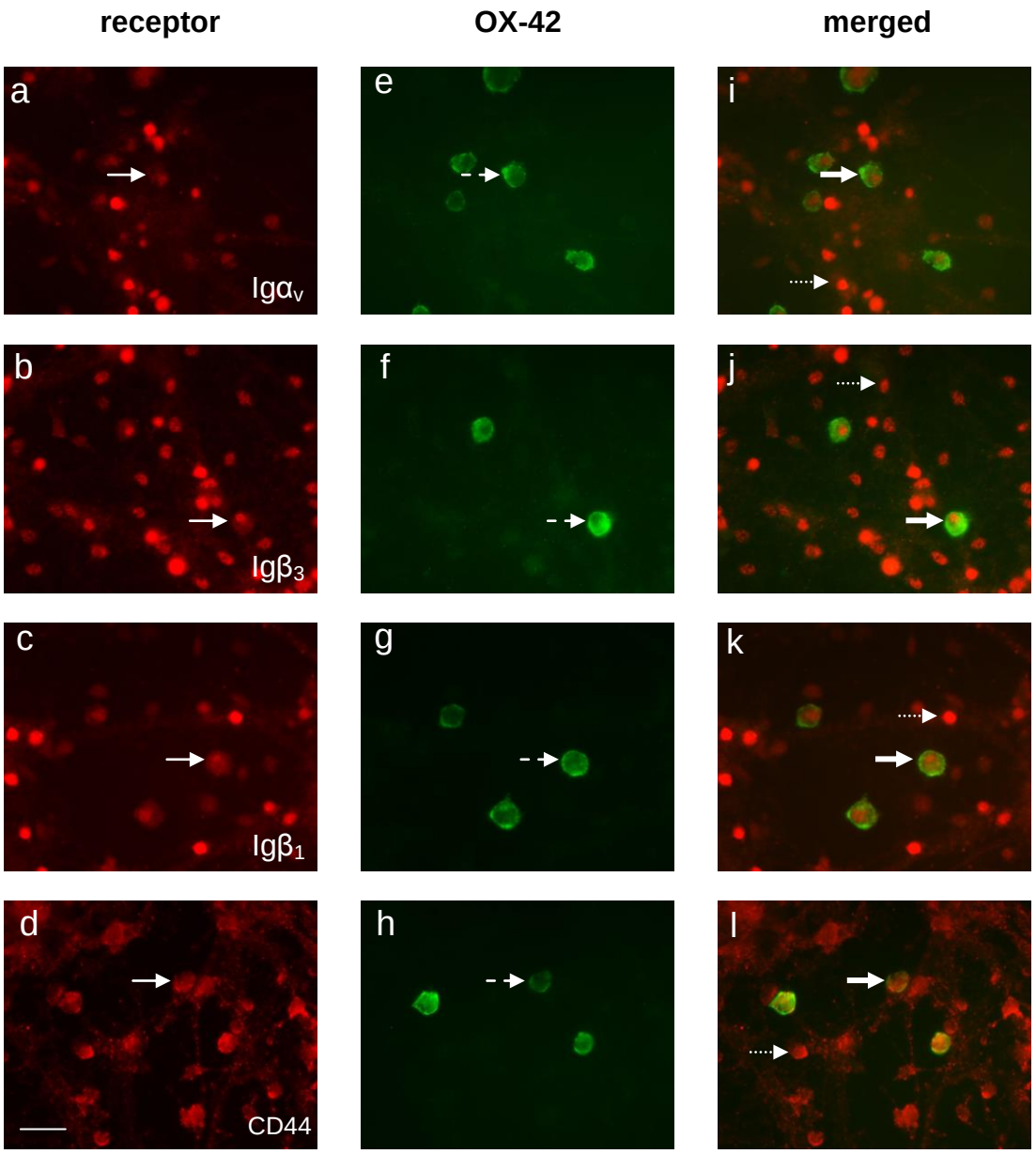

Figure 4.5 Expression of Igα$_v$, Igβ$_3$, Igβ$_1$ and CD44 receptors in OX-42 positive cells in VM cultures.

Representative photomicrographs showing Igα$_v$, Igβ$_3$, Igβ$_1$ or CD44 receptor immunoreactivity (red; a-d; →), OX-42 immunoreactivity (green; e-h, - -→), cells expressing both OX-42 and receptor (i-l; ➡) and cells expressing receptor but not OX-42 (i-l; ····►) in VM cultures. Magnification x40, scale bar = 10μm and is representative of all images.

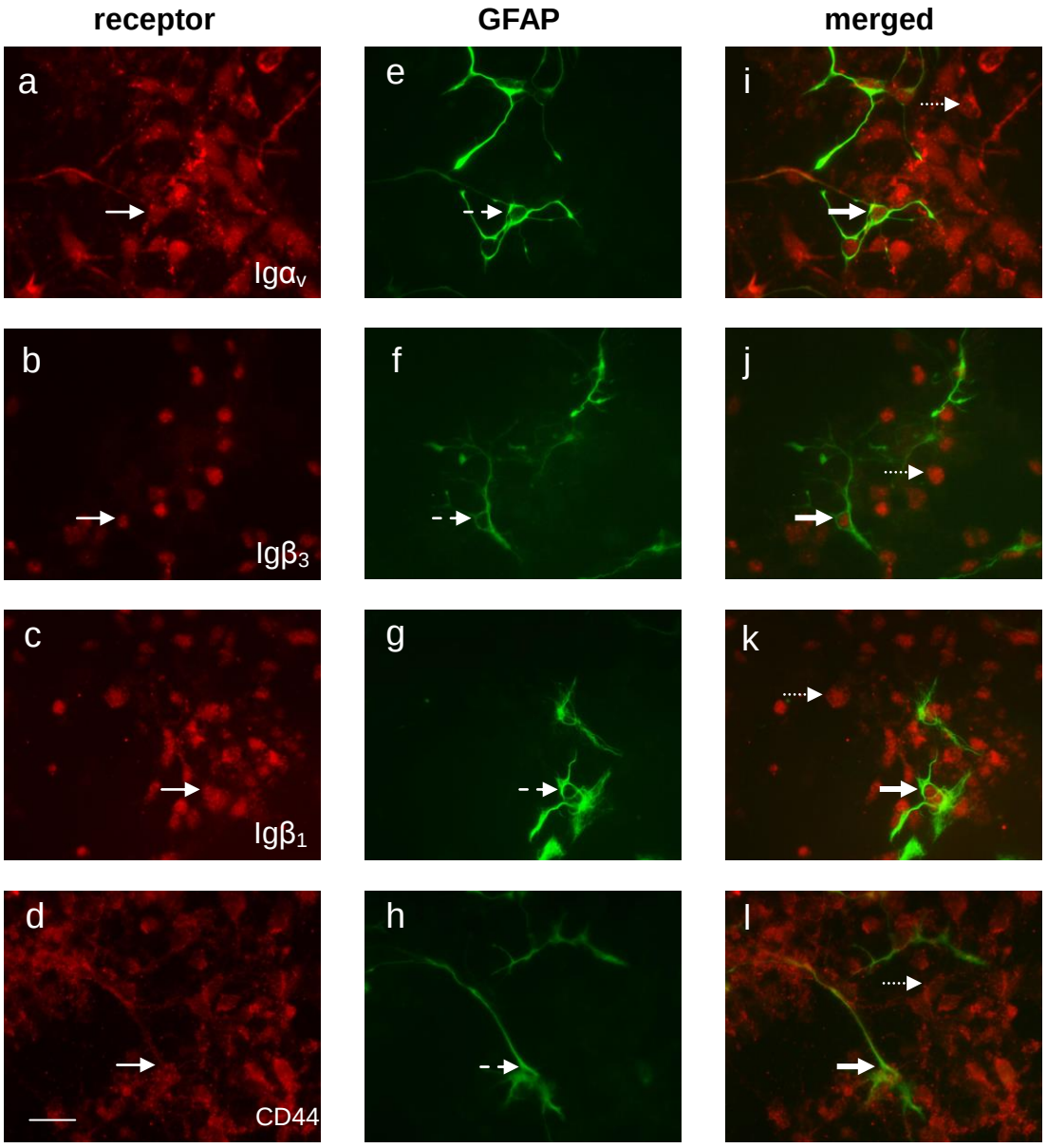

Figure 4.6 Expression of Igα$_v$, Igβ$_3$, Igβ$_1$ and CD44 receptors in GFAP positive cells in VM cultures.

Representative photomicrographs showing Igα$_v$, Igβ$_3$, Igβ$_1$ or CD44 receptor immunoreactivity (red; a-d; →), GFAP immunoreactivity (green; e-h; - -→), cells expressing both GFAP and receptor (i-l; ➡) and cells expressing receptor but not GFAP (i-l; ····►) in VM cultures. Magnification x40, Scale bar = 10μm and is representative of all images.

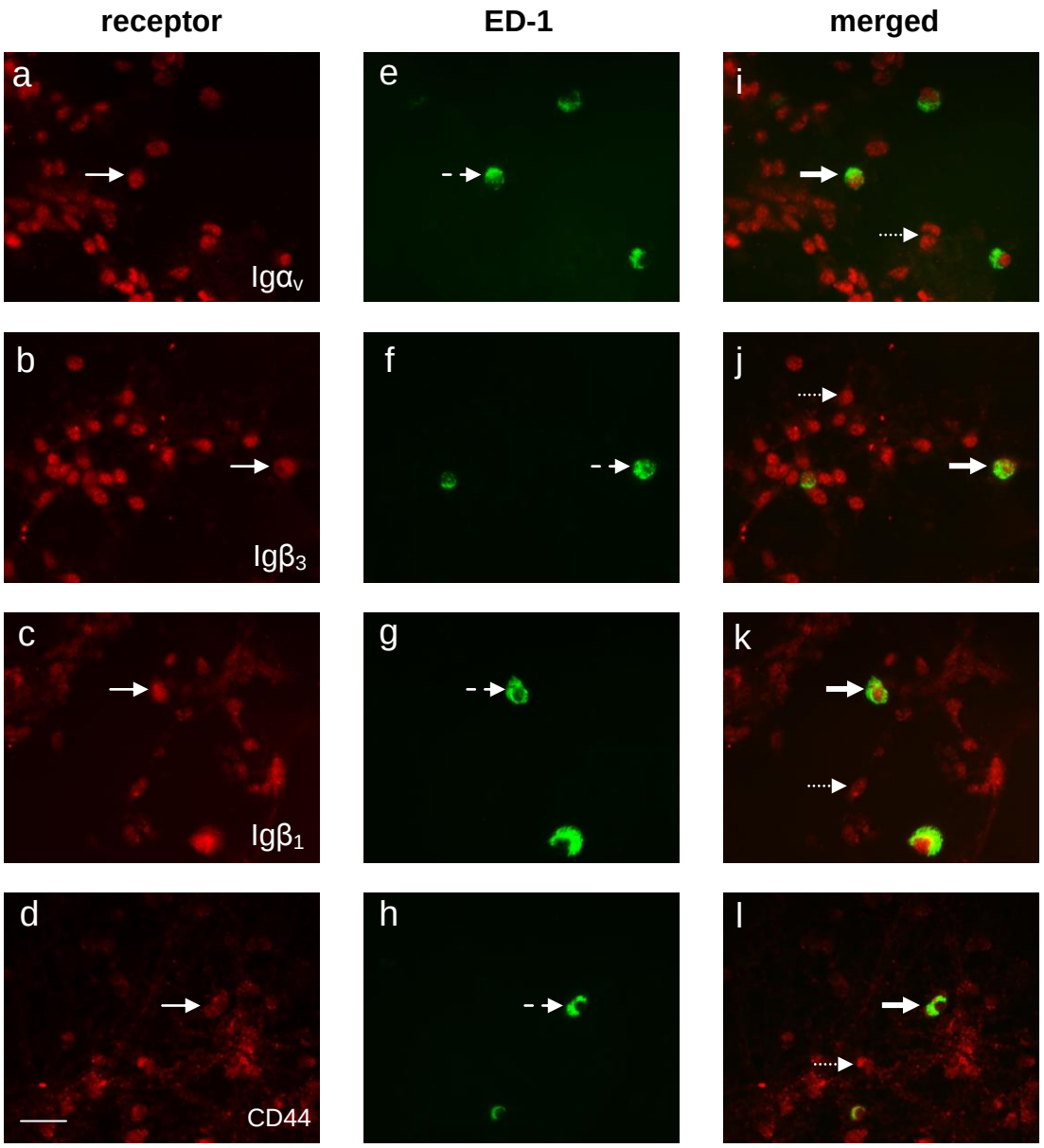

Figure 4.7 Expression of Igα$_v$, Igβ$_3$, Igβ$_1$ and CD44 receptors in ED-1 positive cells in VM culture.

Representative photomicrographs showing Igα$_v$, Igβ$_3$, Igβ$_1$ and CD44 receptors immunoreactivity (red; a-d; →), ED-1 immunoreactivity (green; e-h; - →), cells expressing both ED-1 and receptor (i-l; ➡) and cells expressing receptor but not ED-1 (i-l; ⋯►) in VM cultures. Magnification x40, Scale bar = 10μm and is representative of all images.

4.3.2 Determination of the EC_{50} concentration of MPP^+ in VM cultures

In order to determine the EC_{50} concentration of MPP^+ in VM cultures, MPP^+ (final concentration, 0.1-800µM) was added to cells at DIV 4, the cells were fixed 24hr later and the number of remaining TH immunopositive cells determined. As expected, MPP^+ produced a concentration-dependent loss of TH-positive neurones from VM cultures (Figure 4.8). The EC_{50} concentration obtained was 20±16µM while the highest MPP^+ concentration used (800µM) caused 89% dopaminergic cell loss (Figure 4.8).

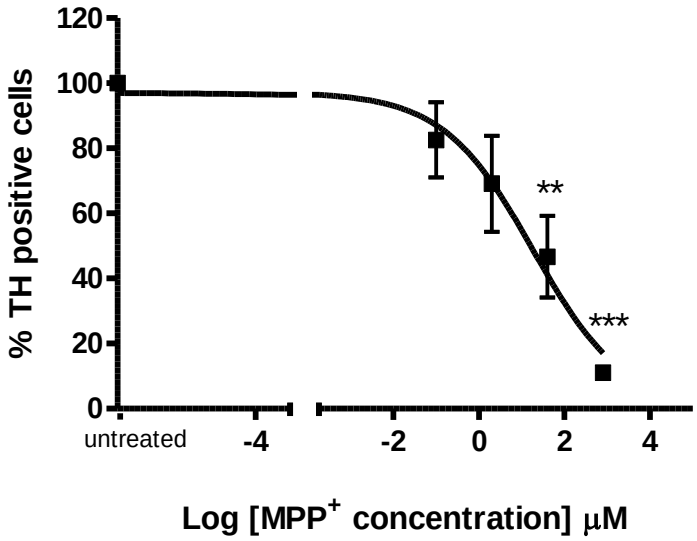

Figure 4.8 The effect of MPP^+ on the number of TH-positive cells in VM cultures.

VM cultures were treated with MPP^+ (0.1-800µM) for 24hr. Cells were immunostained for TH and the number of remaining TH-positive cells were counted and displayed as percentage of TH positive cells compared to untreated cells (=100%). Data are expressed as mean ±SEM (n=4) and analysed by non-linear regression, EC_{50} = 20±16µM. **p<0.01, *** p<0.001 compared to untreated control (one-way ANOVA followed by Newman Keuls test).

4.3.3 The effect of OPN treatment on MPP^+ induced loss of dopaminergic cells in VM cultures

In order to determine the effect of OPN pre-treatment on MPP^+ induced TH positive cell loss in VM cultures, cells were treated with OPN (1-100ng/ml) 24hr prior to treatment with MPP^+ at the pre-determined EC_{50} concentration (20µM). OPN treatment alone did not alter the number of TH immunopositive cells in VM cultures (Figure 4.9). As expected, MPP^+ treatment alone produced 48% loss of TH immunopositive cells. However, pre-treatment with OPN (1-100ng/ml) significantly protected dopaminergic cells of the VM culture from MPP^+ toxicity in a concentration-dependent manner (Figure 4.9). Complete protection was achieved at concentrations greater than 10ng/ml.

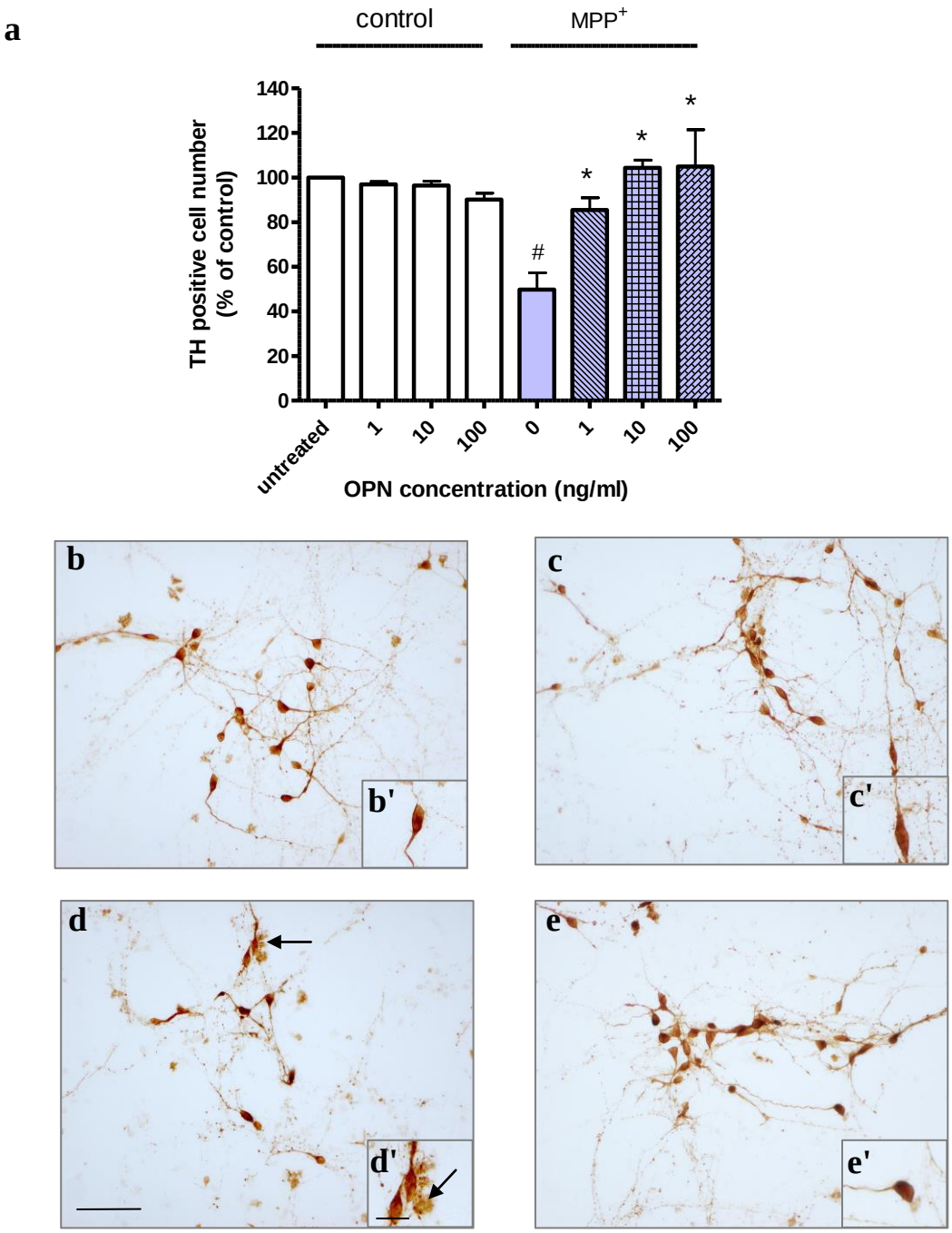

Figure 4.9 The effect of OPN treatment on MPP$^+$ induced dopaminergic cell death in VM culture cells.

VM cultures were treated with OPN (1-100ng/ml) 24hr prior to treatment with MPP$^+$ (20µM). (a) TH immunoreactive cells were counted and displayed as percentage of TH positive cells compared to untreated cells (=100%). Data are expressed as mean ±SEM (n=3). * $p<0.01$ compared to MPP$^+$-only treated cells, # $p<0.01$ compared to untreated control (two-way ANOVA followed by Newman Keuls test). (b-e) Representative photomicrographs showing TH immunostaining in (b) control, (c) OPN (10ng/ml) treated, (d) MPP$^+$ treated and (e) MPP$^+$ and OPN (10ng/ml) treated VM culture cells. Arrows show dead cells. Magnification x20, scale bar in (d) = 200µM and is representative of all images. Scale bar in (d') = 50µM and is representative of all insets.

4.3.4 Investigation of the mechanism of neuroprotection by OPN in VM cultures

4.3.4.a The effect of a 15mer peptide fragment of OPN containing the RGD integrin binding domain on MPP^+ induced dopaminergic cell death

In order to investigate whether a small sequence of OPN comprising the integrin binding domain RGD was responsible for the protective effect seen in Section 4.3.3, a 15-mer peptide fragment comprising the integrin-binding domain of OPN was tested for neuroprotection in VM cultures. Treatment with OPN fragment alone did not alter the number of TH positive cells present in the culture (Figure 4.10). As expected, treatment with MPP^+ produced a 59% decrease in the number of TH positive neurones. Pre-treatment with OPN fragment protected dopaminergic neurones from MPP^+-induced cell death in a concentration-dependent manner, although complete protection was not achieved (Figure 4.10).

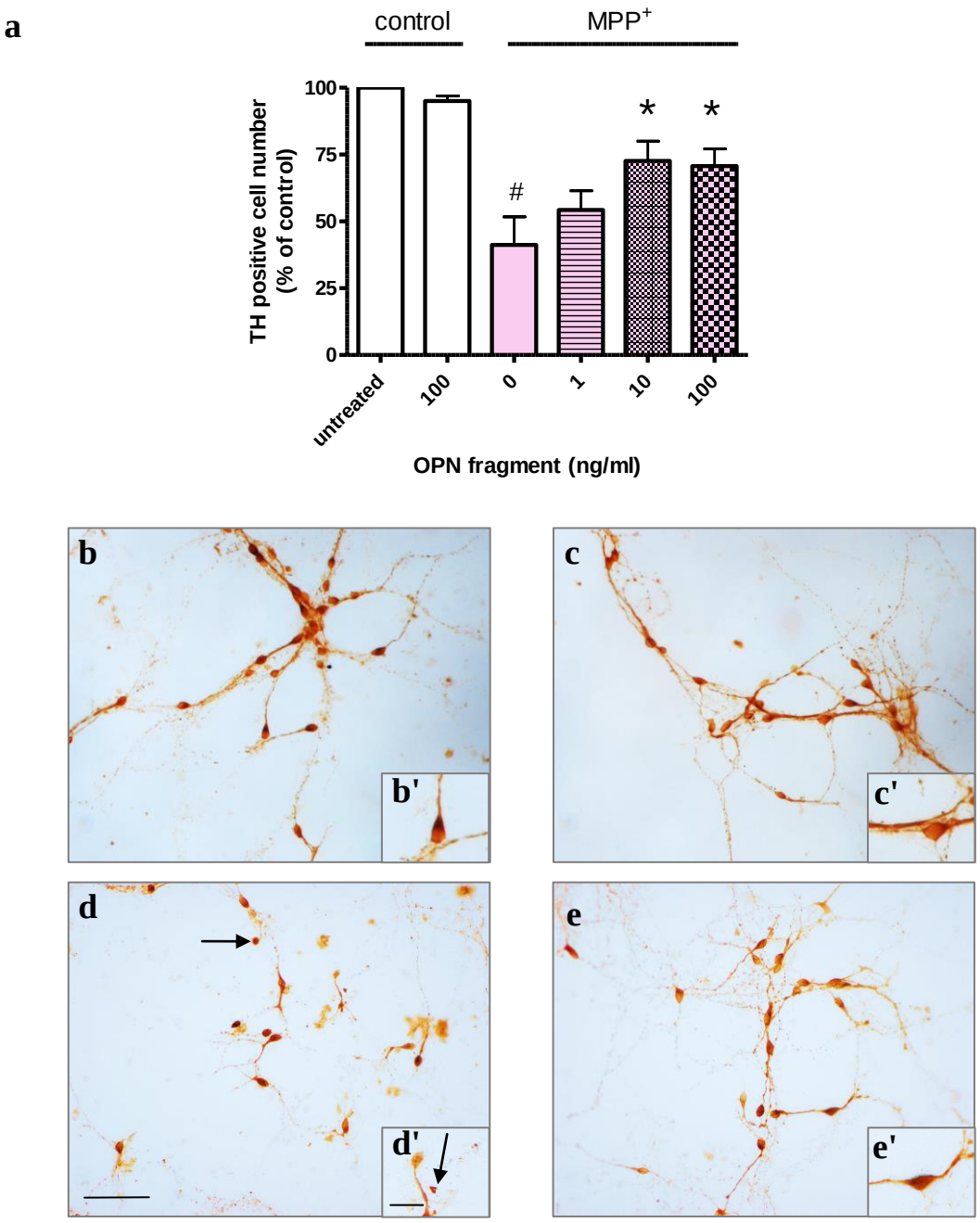

Figure 4.10 The effect of OPN treatment on MPP^+-induced dopaminergic cell death in VM culture cells.

VM cultures were treated with OPN fragment (1-100ng/ml) 24hr prior to treatment with MPP^+ (20µM). (a) TH immunoreactive cells were counted and displayed as percentage of TH positive cells compared to untreated cells (=100%). Data are expressed as mean ±SEM (n=3). # $p<0.01$ compared to untreated control, * $p<0.01$ compared to MPP^+-only treated cells (One-way ANOVA followed by Newman Keuls test). (b-e) Representative photomicrographs showing TH immunostaining in (b) control, (c) OPN fragment (10ng/ml) treated, (d) MPP^+ treated and (e) MPP^+ and OPN fragment (10ng/ml) treated VM culture cells. Arrows show dead cells. Magnification x20, scale bar in (d) = 200µM and is representative of all images. Scale bar in (d') = 50µM and is representative of all insets.

4.3.4.b The effect of integrin receptor inhibitors on neuroprotective effects of OPN

To investigate whether protective effects of OPN shown previously (Section 4.3.3) are mediated via integrin receptors, cells were incubated with non-selective peptide antagonists of integrin receptors, shown previously to inhibit binding to integrin receptors (Meller *et al.*, 2005; Smith *et al.*, 1996) before performing neuroprotection experiments.

RGDS

Integrin receptor antagonist RGDS alone did not alter the number of TH positive cells in the VM culture (Figure 4.11). As expected, MPP^+ (20µM) induced a 58% loss of dopaminergic neurones, and treatment with OPN (10ng/ml) protected TH positive cells from MPP^+ toxicity (Figure 4.11). Treatment of cells with RGDS (1-100ng/ml) 30min before OPN (100ng/ml) prevented the protective effect of OPN in a concentration-dependent manner, with significant effects at 10ng/ml and 100ng/ml (Figure 4.11). In fact, complete inhibition was observed at 10ng/ml and 100ng/ml (Figure 4.11).

GRGDSPK

Integrin receptor antagonist GRGDSPK alone did not have an effect on the number of TH positive cells in the VM culture (Figure 4.11). As expected, MPP^+ (20µM) alone caused 67% loss of dopaminergic neurones, and treatment with OPN (10ng/ml) protected TH positive cells from MPP^+ toxicity (Figure 4.11). Treatment of cells with GRGDSPK (1-100ng/ml) 30min before OPN (1-100ng/ml) inhibited the protective effect of OPN in a concentration dependent manner, with significant effect and complete inhibition at 100ng/ml (Figure 4.11).

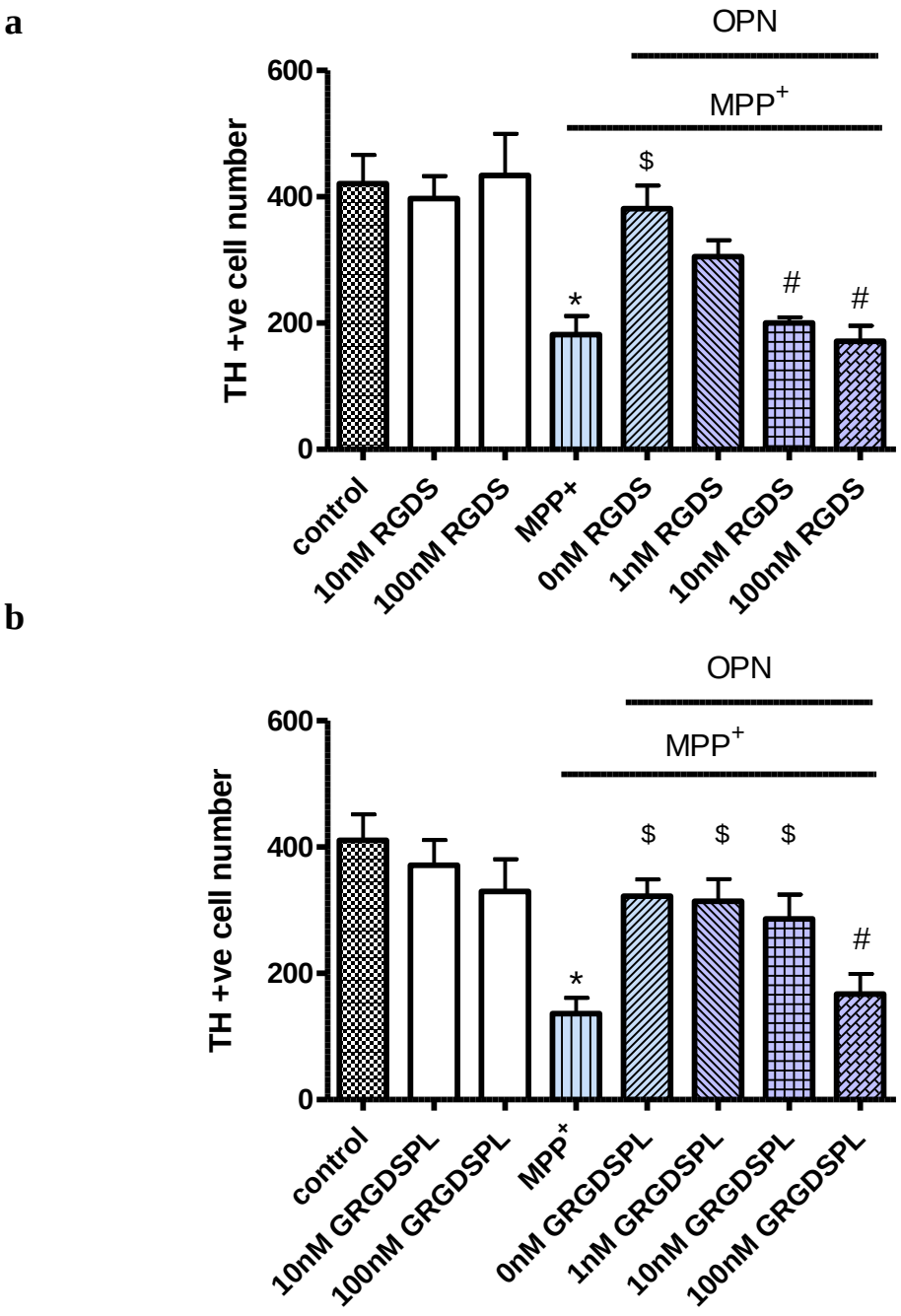

Figure 4.11 Effects of integrin peptide inhibitors on the neuroprotective action of OPN

VM cultures were treated with: (a) RGDS (1-100nM) or (b) GRGDSP (1-100nM) for 30 min before treatment with OPN (100ng/ml). After 24hr, cells were treated with MPP^+ (20µM) and after a further 24hr, the number of TH positive cells was determined. Data are expressed as mean ±SEM (n=3). * $P<0.05$ compared to control, $ $P<0.05$ compared to MPP^+-treated cells, # $P<0.05$ comapred to OPN treated cells treated with MPP^+ (one-way ANOVA followed by Newman Keuls test).

4.3.4.c The effect of OPN on the number of inflammatory cells in MPP^+-treated VM cultures

In order to determine whether protective effects of OPN against MPP^+ induced TH positive cell death was mediated via an action on inflammatory cells, the effect of MPP^+ and/or OPN treatment on the number of OX-42, OX-6, GFAP and ED-1 positive cells was investigated.

OX-42 and OX-6

In untreated control cultures, the number of OX-42 positive cells was 117 cell/10mm^2 (Figure 4.12). Treatment of the VM culture with OPN (10ng/ml) increased the number of OX-42 positive cells in the culture to 198 cell/10mm^2. MPP^+ (20μM) treatment also induced a similar increase in the number of OX-42 cells in the VM culture (171 cell/10mm^2). Treatment of the VM culture with OPN (10ng/ml) 24hr before MPP^+ (20μM) treatment caused an increase in the number of OX-42 positive cells (196 cell/10mm^2) which was not different to that seen after OPN or MPP^+ alone (Figure 4.12). OPN and/or MPP^+ treatment did not change the morphology of OX-42 positive cells (Figure 4.12). No microglial activation occurred following any of the treatments, as no OX-6 positive staining was obtained for any treatment condition (Data not shown).

GFAP

Untreated control cultures contained 58 cell/10mm^2 GFAP positive cells. Treatment of the VM culture with OPN (10ng/ml) or MPP^+ (20μM) did not induce a change in the number of GFAP positive cells in the culture (Figure 4.13). Similarly, treatment of the VM culture with OPN (10ng/ml) 24hr before MPP^+ (20μM) did not affect the number of GFAP positive cells (Figure 4.13). None of the treatment conditions changed the morphology of GFAP positive cells (Figure 4.13).

ED-1

In untreated control cultures, there was 163 cell/10mm^2 ED-1 positive cells (Figure 4.14). The cells appeared round and showed strong staining of cell bodies but not processes. Treatment of the VM culture with OPN (10ng/ml) or MPP^+ (20μM) did not induce a significant change in the number of ED-1 positive cells in the culture or in morphology (Figure 4.14). Similarly, treatment of the VM culture

with OPN (10ng/ml) 24hr before MPP^+ (20µM) did not affect the number or morphology of ED-1 positive cells (Figure 4.14).

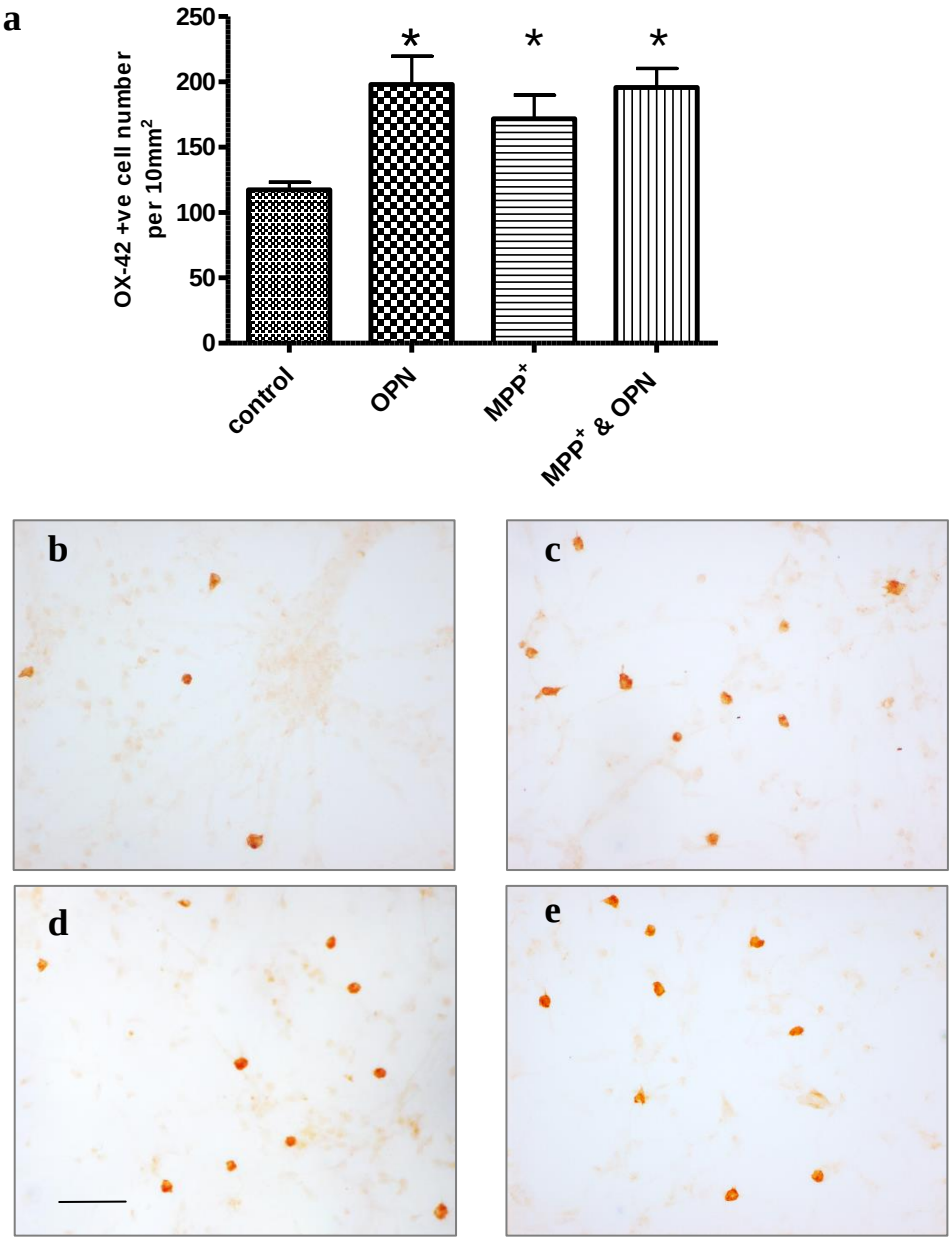

Figure 4.12 The effect of OPN on the number of OX-42 positive glial cells in MPP^+-treated VM culture.

VM cultures were treated with OPN (10ng/ml) or MPP^+ (20µM), alone or in combination, OPN being added 24hr prior to MPP^+. (a) Cells were immunostained with anti-OX42 antibody and the number of OX-42 positive cells was determined. Data represent the number of OX-42 positive cells in $10mm^2$ and are expressed as mean ±SEM (n=3). * $P<0.05$ compared to control (two-way ANOVA followed by Newman Keuls test). (b-e) Representative photomicrographs showing OX-42 immunostaining in (b) untreated control, (c) OPN treated, (d) MPP^+ treated and (e) MPP^+ and OPN treated VM cultures. Magnification x20, scale bar = 200µM and is representative of all images.

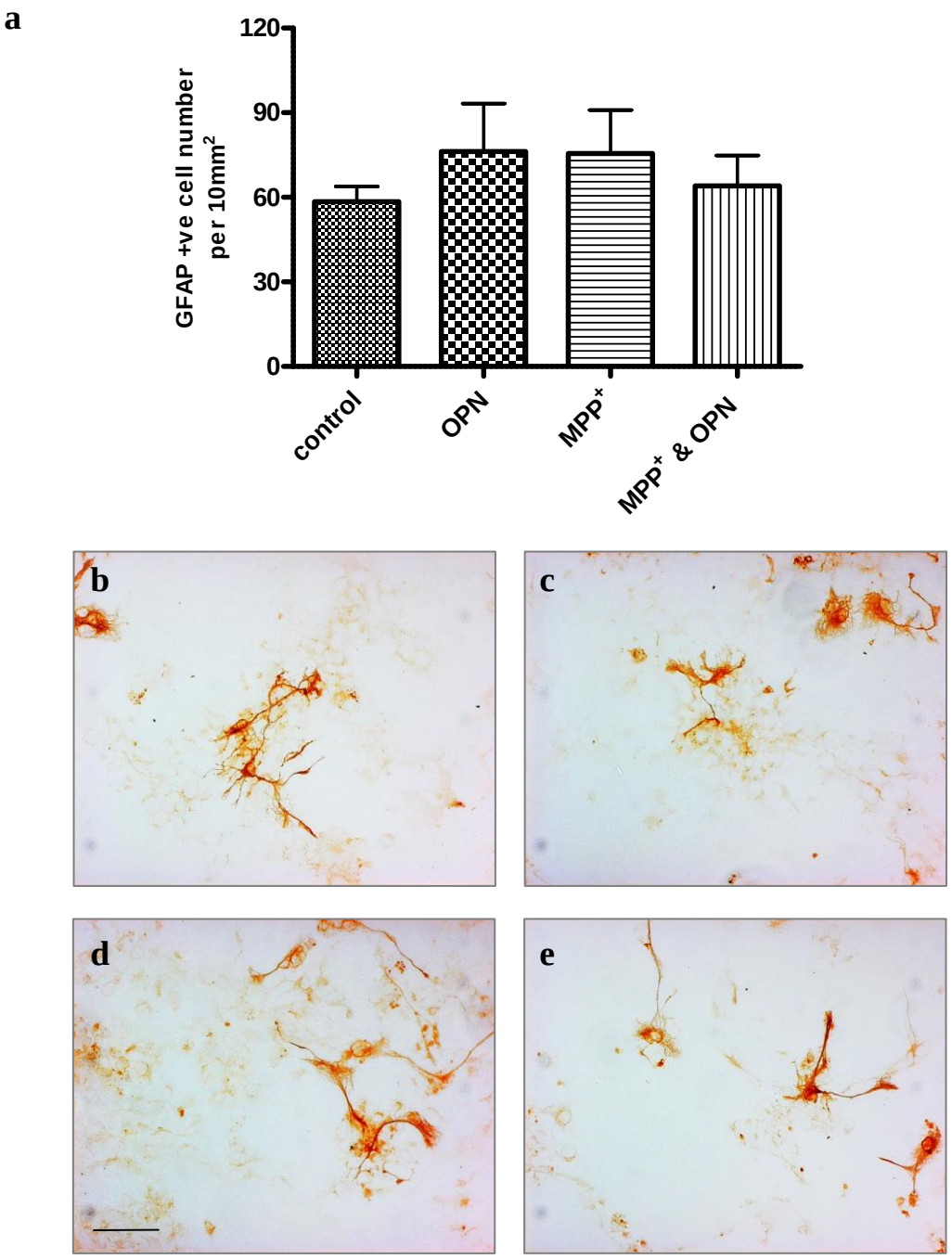

Figure 4.13 The effect of OPN on the number of GFAP positive glial cells in MPP^+-treated VM culture.

VM cultures were treated with OPN (10ng/ml) or MPP^+ (20µM), alone or in combination, OPN being added 24hr prior to MPP^+. (a) Cells were immunostained with anti-GFAP antibody and the number of GFAP positive cells was determined. Data represent the number of GFAP positive cells in $10mm^2$ and are expressed as mean ±SEM (n=3). Data were analysed using two-way ANOVA. Representative photomicrographs showing GFAP immunostaining in (b) untreated control, (c) OPN treated, (d) MPP^+ treated and (e) MPP^+ and OPN treated VM cultures. Magnification x20, scale bar = 200µM and is representative of all images.

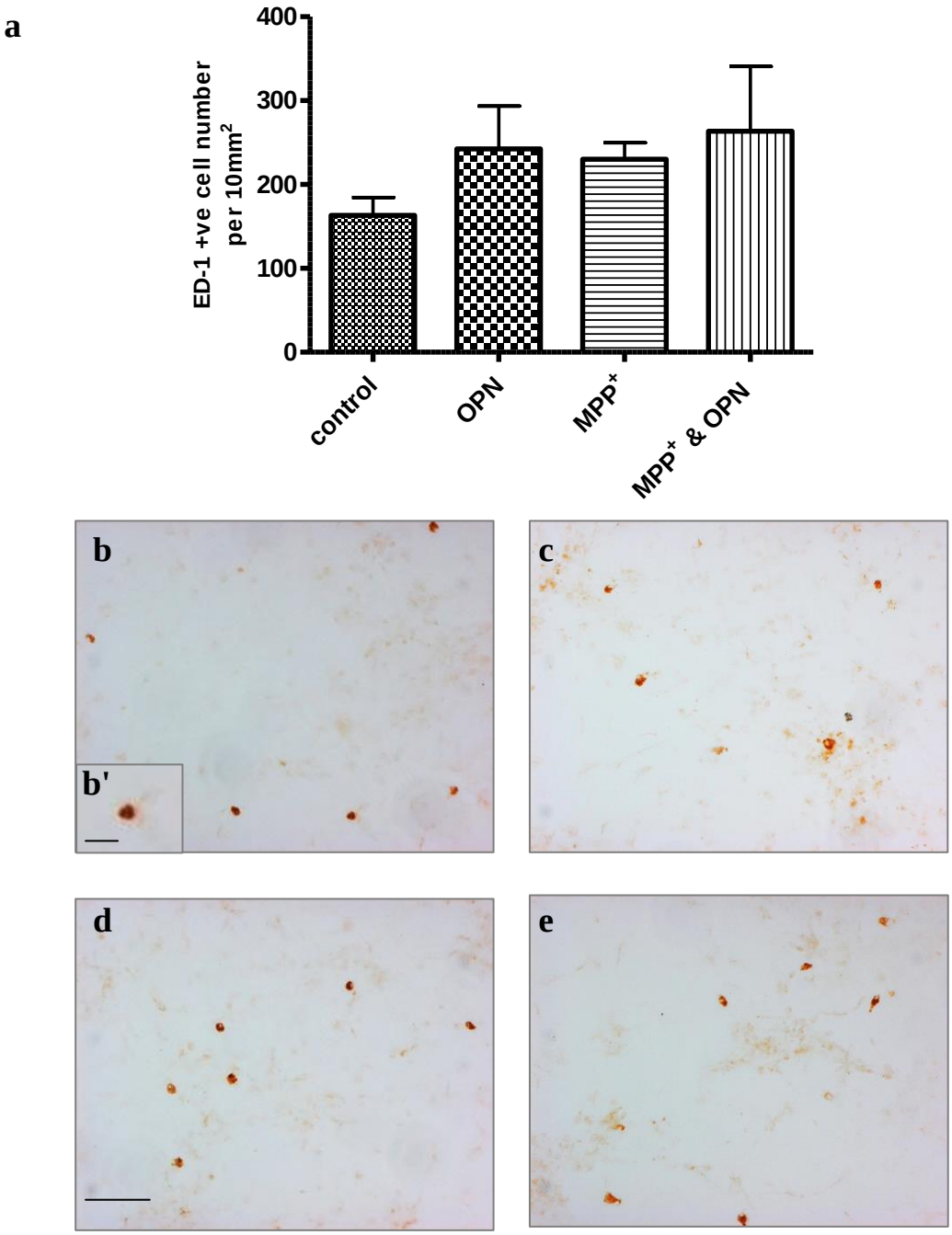

Figure 4.14 The effect of OPN treatment on the number of ED-1 positive cells in MPP^+ treated VM culture.

VM cultures were treated with OPN (10ng/ml) or MPP^+ (20µM), alone or in combination, OPN being added 24hr prior to MPP^+. (a) Cells were immunostained with anti-ED-1 antibody and the number of ED-1 positive cells was determined. Data represent the number of ED-1 positive cells in $10mm^2$ and are expressed as mean ±SEM (n=3). Data were analysed using two-way ANOVA. (b-e) Representative photomicrographs showing GFAP immunostaining in (b) untreated control, (c) OPN treated, (d) MPP^+ treated and (e) MPP^+ and OPN treated VM cultures. Magnification x20, scale bar in (d) = 200µM and is representative of all photomicrographs. Scale bar in (b') = 50µM.

4.4 Discussion

OPN did not prevent MPP^+ or H_2O_2 induced cell death in immortalised dopaminergic cell lines (Chapter 3), although a fragment of OPN has recently been shown to be neuroprotective in VM cultures (Iczkiewicz *et al.*, 2010). This has led to an investigation of OPN effects on dopaminergic neurones in primary VM culture. Therefore, in this study, it was hypothesized that OPN protects against toxin induced cell death in primary VM culture and that this effect is mediated via integrin receptors and CD44 and through an action on glial cells.

4.4.1 Characterisation of the VM culture

Between DIV 3 and 5, the VM cultures were comprised of approximately 5% TH immuopositive cells and 50% GAD immunopositive cells. These results support previous reports of 2-5% TH immunopositive and 48% GAD immunopositive cells in VM cultures (Cheung *et al.*, 1997; Gilbert *et al.*, 2003; Johansson *et al.*, 2003). The VM cultures also contained OX-42 positive microglia, GFAP positive astrocytes and ED-1 positive macrophages. This confirms that primary VM cultures contain not only dopaminergic neurones but also other cell types most notably glial cells which may be critical for the protective effects of OPN.

OPN binds to many integrin receptors and to the CD44 receptor in order to mediate a range of functions (Chapter 1). Integrins α_v, β_3 and β_1 as well as the CD44 receptor have been shown to mediate pro-survival actions of OPN (Caers *et al.*, 2006; Lee *et al.*, 2007; Lin *et al.*, 2001; Scatena *et al.*, 1998). In this study, for the first time, dopaminergic neurones in VM culture were shown to express $Ig\beta_3$, $Ig\beta_1$ and CD44 and the expression of $Ig\alpha_v$ was confirmed (Chao *et al.*, 2003). This demonstrates that dopaminergic neurones in VM culture express receptors that may mediate pro-survival effects of OPN. Thus, they may provide an appropriate test bed for investigating neuroprotective effects of OPN. In addition, dopaminergic neurons were shown to express OPN. Although expression of OPN constituvely in cell lines did not affect their vulnerability to toxins (Chapter 3), a recent study in these laboratories showed different results in VM culture. In this study, treating VM cultures with an anti-OPN antibody rendered dopaminergic cells more vulnerable to MPP^+ and LPS toxicity, exacerbating more cell loss (Iczkiewicz *et al.*, 2010). In

fact, treatment of VM cultures with anti-OPN antibody alone induced dopaminergic cell loss (Iczkiewicz *et al.*, 2010).

4.4.2 OPN protects dopaminergic neurons in VM cultures from MPP^+-induced cell loss

The susceptibility of dopaminergic neurones in VM culture to MPP^+ was confirmed, as it produced a concentration dependent decrease in TH positive cell number with an EC_{50} = 20µM. This concentration is comparable to the range used in previous studies (20-50µM) (Grammatopoulos *et al.*, 2007; McNaught *et al.*, 1999a; Outeiro *et al.*, 2007). It is noteworthy that the EC_{50} concentration of MPP^+ in VM culture is about 100 fold lower than that in cell lines (2mM). This suggests that cell lines may be different to primary neurones as they are more resistant to toxins and may thus be less sensitive to protective agents.

Importantly, OPN (1-100ng/ml) pre-treatment of VM cultures prevented MPP^+ toxicity with complete protection of dopaminergic neurons at concentrations (10 and 100ng/ml). This is in line with a previous report of the ability of a 15-mer fragment of OPN to protect dopaminergic neurons against MPP^+ toxicity (Iczkiewicz *et al.*, 2010). However, these results are completely different to those obtained in SH-SY5Y and N1E-115 cell lines despite the fact that they express $Ig\alpha_v$, $Ig\beta_3$ and $Ig\beta_1$ and CD44 receptors. In fact, exogenous OPN treatment did not protect SH-SY5Y and N1E-115 cell lines from MPP^+ or H_2O_2 induced cell death nor did transfection of SH-SY5Y cells with the protein (Chapter 3). These findings perhaps indicate that the lack of protective effect in cell lines was due to differences between neuronal cell lines and VM culture. It is possible that, as anticipated, presence of glial cells in the VM culture but not cell lines can affect regulation of OPN function and this is discussed in Section 4.4.4. Also, post translational modifications of OPN such as phosphorylation and proteolytic cleavage may be different in VM culture, thereby facilitating interaction between protein and receptor and mediating protective mechanisms. These post translational modifications have been shown to affect receptor binding and function of OPN as discussed in Chapter 3.

4.4.3 The role of integrin receptors in the neuroprotective effect of OPN

Non-specific peptide inhibitors of integrin receptors (RGDS and GRGDSPK) prevented the protective effects of OPN in VM cultures exposed to MPP^+. This suggests that the protective effect of OPN was mediated through integrin receptors and this is in line with other studies where pro-survival effects of OPN were mediated via integrin receptors (Caers *et al.*, 2006; Lee *et al.*, 2007; Lin *et al.*, 2001; Lin *et al.*, 2000; Scatena *et al.*, 1998). The role of CD44 receptor in protective actions of OPN could not be investigated as there is no commercially available neutralising anti-CD44 antibody suitable for use in cells of rat origin. In subsequent studies this could potentially be investigated using silencing RNA. The fact that integrin inhibitors fully inhibited protection by OPN does not necessarily rule out a role for the CD44 receptor as binding to CD44 requires OPN to first bind $Ig\beta_1$ (Katagiri *et al.*, 1999). In the same study, however, binding to $Ig\beta_1$ was not RGD dependent (Katagiri *et al.*, 1999).

In addition, a 15-mer fragment of OPN containing the RGD binding domain, which binds to $\alpha_v\beta_3$, $\alpha_v\beta_1$, $\alpha_v\beta_5$, $\alpha_5\beta_1$ and $\alpha_8\beta_1$ integrin receptors (Attur *et al.*, 2000; Denda *et al.*, 1998; Hu *et al.*, 1995), retained the neuroprotective properties of OPN in the VM culture. This is in line with the previous report of the protective effect of this fragment in MPP^+ and LPS treated VM cultures (Iczkiewicz *et al.*, 2010). However, the number of TH positive cells was lower than untreated control, indicating that MPP^+ was still inducing some cell death in the presence of the 15-mer fragment of OPN. Therefore, this segment of OPN did not fully protect dopaminergic neurones suggesting that other parts of the protein may be contributing to the pro-survival effects of OPN. For instance the CD44 receptor, which binds a site of the protein, not included in the fragment, has been shown to mediate OPN-induced cell survival in different cell lines (Lin *et al.*, 2001; Lin *et al.*, 2000). In fact, unpublished data have shown a protective effect of a fragment of OPN containing the CD44 binding domain in toxin treated VM culture (Iczkiewicz *et al*. 2010). In addition, binding to CD44 is suggested to regulate OPN binding to integrin receptors possibly via activating integrins through an outside-in mechanism and increasing cell survival (Lee *et al.*, 2007). Another possibility is that the full length protein may have enhanced binding to receptors via conformational changes to the protein binding site.

Inhibition of the protective effect of OPN against MPP^+ toxicity by integrin antagonists and the positive effect of the RGD containing fragment of OPN suggests that protective effects of OPN protein are at least partially due to binding of integrin receptors. Clearly, integrin receptors are involved in the neuroprotective effect of OPN in VM culture but whether this effect requires the involvement of glial cells is not yet clear.

4.4.4 The role of glial cells in OPN-induced neuroprotection

Immunohistochemical investigation showed that VM culture contains microglia, astrocytes and ED-1 positive macrophages. This confirms results of previous studies showing that the VM culture comprises OX-42 and GFAP positive glia (Cheung *et al.*, 1997; Gilbert *et al.*, 2003). In fact, in the present study, glial cells expressed receptors shown to mediate protective actions of OPN, $Ig\alpha_v$, $Ig\beta_3$, $Ig\beta_1$ and CD44. This suggests that OPN may have effects on these cells that contribute to neuroprotection, such as increased production of neurotrophic factors.

Neither MPP^+ nor OPN had an effect on the number of astrocytes or macrophages present in the VM culture. This is the first investigation of OPN's effect on these cells in VM cultures, while MPP^+ was not previously reported to affect the number of GFAP positive astrocytes or ED-1 positive macrophages in VM culture. OX-6 positive activated microglia could not be detected in the VM cultures under any treatment condition. In one study, however, MPP^+ induced an increase in the number of OX-6 positive microglia (Joglar *et al.*, 2009) but this was after four days of MPP^+ treatment. Unlike the reported effects of MPTP *in-vivo* (Breidert *et al.*, 2002; Chung *et al.*, 2010), MPP^+ did not have an effect on astrocytes or macrophages in VM culture suggesting that these glial cells may be immature and therefore may not act like mature glia in adult rat SN.

MPP^+ caused an increase in the number of OX-42 positive microglia in the VM culture in agreement with previous reports (Henze *et al.*, 2005). Interestingly, OPN also caused an increase in the number of OX-42 positive microglia in the VM culture and this is in line with previous reports of the ability of OPN to recruit inflammatory cells as part of its function (Ellison *et al.*, 1999; Lampe *et al.*, 1991; O'Regan *et al.*, 1999). However, pre-treatment of cells with OPN 24hr before MPP^+ treatment caused an increase in the number of microglial cells in the VM culture similar to that

caused by either agent alone. It is therefore not clear whether this effect is produced by OPN, MPP^+ or both. It is possible that microglia reached maximal proliferation capacity so treatment with both agents could not have an additive effect.

Although OPN protected the VM culture from MPP^+ induced dopaminergic cell loss, it did not appear to affect the MPP^+ induced increase in the number of microglial cells and may in fact be contributing to this increase. Therefore, OPN may be acting directly on dopaminergic neurones instead, and this is supported by the fact that dopaminergic neurones in the VM cultures were shown to express receptors involved in OPN's pro-survival actions. However, although cell lines expressed these receptors, OPN did not protect them against toxin-induced cell death (Chapter 3) This may suggest that expression of receptors is not sufficient to allow protection by OPN, effects on glial cells or specific modification of OPN by VM cultures may also be necessary.

It was previously shown that MPP^+ induces iNOS up-regulation in VM cultures (Du *et al.*, 2001). Therefore, it is conceivable that OPN acts by decreasing NO levels from the VM culture by means of inhibiting up-regulation of iNOS as reported previously (Rollo *et al.*, 1996b) but this requires further investigation. Additionally, the increase in microglial cells caused by OPN treatment alone may be a protective action, since it was recently shown in unpublished studies from our laboratories that both OPN and MPP^+ cause an increase in neurotrophic factors released by glial cells (GDNF and BDNF) in VM cultures (Broom, 2011, personal communication). Glial cells in the SN determine the homeostatic control of the neuronal extracellular environment, provide support to neurones through the release of neurotrophic factors, protect neurones against toxic insult and have the ability to counteract oxidative stress (Hirsch, 2000; Montgomery, 1994). In pathological situations, glial cells may play a protective role by releasing trophic factors such as GDNF or BDNF, cleavage of free radicals through glutathione peroxidase or catabolising dopamine by monoamine oxidase B or COMT. They may also contribute to damage by releasing pro-inflammatory cytokines and promoting immune reactions (Barron, 1995; Hirsch, 2000; Hirsch *et al.*, 2003; McGeer *et al.*, 1998; Vila *et al.*, 2001a). It is possible therefore that in this instance, OPN induced proliferation of microglia is not a harmful action particularly as it did not cause microglial activation or cause a decrease in the number of dopaminergic neurones present in culture. It is difficult to

explain the increase in microglia produced by OPN and it is not easy to see why OPN would do this by itself. Since OPN is induced as a response to injury or inflammation (Chapter 1), it may be that adding OPN alone to the culture sends signals that there is injury and hence microglial proliferation occurs.

4.4.5 Conclusion

In conclusion, these data confirm the toxic effect of MPP^+ in VM culture cells and show for the first time the ability of OPN to protect dopaminergic neurones from this toxicity. Protection was mediated via integrin receptors which are expressed on dopaminergic neurones and inflammatory cells, although the role of CD44 remains to be further investigated. OPN induced proliferation of microglial cells but not astrocytes or macrophages and did not induce activation of microglia, so the exact role of glial cells and macrophages in OPN's protective effects is not clear.

Chapter 5 OPN prevents LPS induced nigral dopaminergic cell death in the rat and attenuates microgliosis

5.1 Introduction

In the previous chapter, OPN was shown to protect against MPP^+ induced dopaminergic cell loss in VM cultures and this was mediated via integrin receptors. The protective effect of OPN against MPP^+ induced cell death was accompanied by an increase in the number of microglial cells, but as discussed in Chapter 4, it was difficult to determine their role in OPN's protective effect. OPN is involved in the regulation of inflammatory processes in the periphery and CNS (Chapter 1) and was previously shown to be up-regulated in the SN following supra-nigral LPS injection in rat (Iczkiewicz *et al.*, 2005). OPN may therefore be involved in regulating the inflammatory process in PD. The obvious thing to do, perhaps, would have been to look more specifically into the role of glial cells using LPS in VM culture. However, the ultimate objective of these studies was to determine whether full length OPN is protective in the brain. In addition, a study was recently published using supra-nigral LPS injection in rats as a model of cell death in PD and in fact showing that a 15-mer peptide fragment of OPN is protective (Iczkiewicz *et al.*, 2010). However, it is not known whether full length OPN is equally protective since this has not been investigated previously in experimental models of dopaminergic cell death. Therefore, a more logical step is to focus on the LPS model to investigate the role of OPN in regulating inflammation, but to use it in the SN *in-vivo*. Consequently, the rat LPS model of inflammation in PD was employed to investigate the effects of OPN on inflammatory mediated dopaminergic cell loss.

Whether, in the intact rat SN, evidence can be found for integrin receptors which seem to be important for protection; $Ig\alpha_v$, $Ig\beta_3$, $Ig\beta_1$ (Chapter 4 and Chapter 1) and CD44 (Chapter 1) is to be determined. Although, $Ig\alpha_v$ and CD44 were previously shown to be expressed on dopaminergic neurons of the SN (Chao *et al.*, 2003; Fuxe *et al.*, 1996), a full investigation of $Ig\alpha_v$, $Ig\beta_3$, $Ig\beta_1$ and CD44 receptors and their cellular localisation within the rat SN has not been undertaken. It is also not known whether expression of these receptors is altered following LPS lesioning in the SN and whether it is different in activated glia and infiltrating macrophages. This is an important issue, but from the studies described in Chapter 3, expression of these receptors on cells does not necessarily indicate that OPN is able to exert protective effects.

5.1.1 Hypothesis

OPN protects against inflammation induced cell death of dopaminergic neurons and this is mediated through an interaction with Igα_v, Igβ_3, Igβ_1 or CD44 receptors and an effect on glial cells.

5.1.2 Aims

In order to test this hypothesis, studies with the following aims were performed

- To characterise normal rat SN for the expression of Igα_v, Igβ_3, Igβ_1 and CD44 receptors.
- To confirm the dopaminergic cell loss and reactive gliosis in the LPS model of PD.
- To determine the effect of LPS on the expression of Igα_v, Igβ_3, Igβ_1 and CD44 receptors.
- To determine whether OPN pre-treatment protects nigral dopaminergic neurones from LPS mediated degeneration.
- To determine if the effects of OPN are mediated through changes in the reactive gliosis induced by LPS.

5.2 Methods

OPN was investigated for neuroprotective effects in the LPS model of nigral cell death in PD. LPS, a component of the outer membrane of gram negative bacterial cell wall, is an endotoxin and a potent inducer of inflammation (Burrell, 1990). Administration of LPS into rat SN produces significant loss of dopaminergic neurones due to rapid activation of glial cells (Herrera *et al.*, 2000; Iravani *et al.*, 2005). LPS was shown to cause a persistent and selective degeneration of the dopaminergic neurons of SN, having no effect on GABAergic or serotonergic neurons (Castano *et al.*, 1998). In addition, LPS induced toxicity is not via direct action on neurons but it works via activation of glial cells and inducing the release of pro-inflammatory cytokines such as TNF-α and IL-1β (Liu *et al.*, 2000b). This makes the LPS model a suitable model for studying inflammation mediated neurodegeneration. In addition to the inflammatory reaction, LPS was shown to produce a reduction in glutathione levels in SN indicating that oxidative stress may also be a contributory factor in cell death (Dutta *et al.*, 2008). This model was previously used to investigate potential treatments for PD such as naloxone (Liu *et al.*, 2000a) and minocycline (Tomás-Camardiel *et al.*, 2004).

5.2.1 Investigation of Igα_v, Igβ_3, Igβ_1 and CD44 receptors expression in the normal rat SN

In order to investigate the expression of Igα_v, Igβ_3, Igβ_1 and CD44 receptors in the normal rat SN, a group of male Wistar rats (250-280g, n=5) were deeply anaesthetised with sodium pentobarbital (130mg/kg, i.p.). The rats were transcardially perfused with 0.1M PBS followed by PFA (4%/0.1M PBS) and then brain SN sections prepared as described in Section 2.5.2a. The expression of Igα_v, Igβ_3, Igβ_1 receptors and CD44 in rat brain SN was investigated by immunoperoxidase histochemistry as described in Section 5.2.4 and double labelling immunofluorescence as described in Section 5.2.5. All experiments were performed under Animal (Scientific procedures) act 1986 and approved by King's College Ethical Review panel, Project licence 70/6018.

5.2.2 LPS rat model of PD

Supra-nigral injection of LPS was performed in the rat brain as described in Section 2.5.1. Briefly, male Wistar rats (250-300g, n=6 per group) were anaesthetised with isoflorane (4% in 5%O_2: 95% CO_2) and secured in stereotaxic frame (Kopf instruments, US). LPS (5µg/µl; 2µl) or vehicle (saline, 0.9%; 2µl) were slowly injected over 2min at co-ordinates -4.8mm anterior, +2.0mm lateral and -7.6mm ventral to bregma (Paxinos and Watson, 1986; Figure 5.1). The needle was slowly withdrawn, wounds sutured and animals allowed to recover. Rats were culled at three different time points (1, 3 or 7 days) following surgery and peroxidase immunohistochemistry was performed to determine the effect of supranigral LPS on the expression of TH, OX-6, GFAP and ED-1 as described in Section 5.2.4. Double labelling immunofluorescence was employed to examine the cellular localisation of integrin and CD44 receptors after LPS lesioning as described in Section 5.2.5.

5.2.3 Neuroprotection studies

In order to test the neuroprotective effects of OPN *in-vivo*, recombinant rat OPN (custom made, GenScript; 100ng, 2µl) or vehicle (PBS, 2µL) were injected into the rat SN following the same surgical procedure described for the LPS model in Section 2.5.1 using co-ordinates -4.8mm anterior, +1.8mm lateral and -8.0mm ventral to bregma (Paxinos and Watson, 1986; Figure 5.1). OPN or vehicle were injected either 2 or 24hr before supra-nigral LPS (5µg/µl; 2µl) or vehicle (0.9% saline; 2µl) injections performed as described in Section 5.2.2. Rats were culled 1 week after surgery and peroxidase immunohistochemistry was performed as described in Section 5.2.4 to investigate the effects of OPN pre-treatment on the LPS-induced nigral dopaminergic cell loss and gliosis.

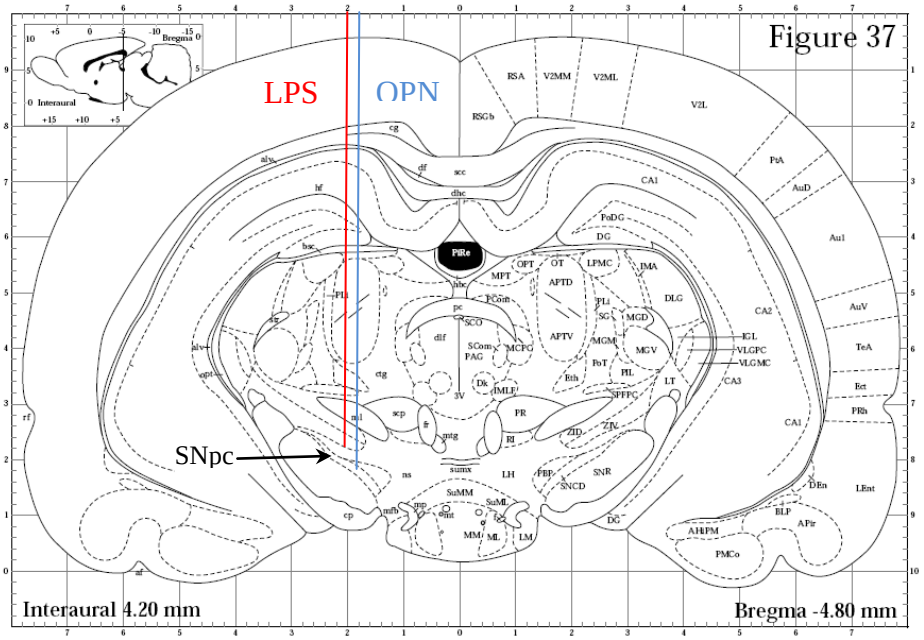

Figure 5.1 Schematic diagram of LPS and OPN injection co-ordinates.

OPN was injected into the SN at co-ordinates -4.8mm anterior +1.8mm lateral and -8.0mm ventral to bregma. LPS was injected supra-nigrally at co-ordinates -4.8mm anterior, +2.0mm lateral and -7.6mm to bregma (Paxinos and Watson, 1986).

5.2.4 Peroxidase immunohistochemistry in rat SN

Peroxidase immunohistochemistry was used to characterise the normal and LPS injected SN for the expression of Igα_v, Igβ_3, Igβ_1 and CD44 and to study the changes induced by LPS and OPN on the expression of TH, OX-6, ED-1 and GFAP. Peroxidase immunohistochemistry was performed as described in detail in Section 2.5.2. Briefly, SN sections were incubated with primary antibodies (Table 2.5) overnight at room temperature. After two washes in 0.1M PBS, sections were incubated with biotinylated secondary antibodies (Table 2.6) for one hour. The sections were then washed twice in 0.1M PBS followed by incubation in the Avidin Biotin complex (1:200) for one hour. For visualisation of results, sections were incubated with DAB (0.05% in 0.05M Tris HCl; Ph 7.4) for 5min then H_2O_2 (0.01% final concentration) was added and the sections incubated for 1-5min. Sections were dehydrated and coverslipped as described in Section 2.5.2.b.

5.2.5 Double labelling fluorescence immunohistochemistry in rat SN tissue sections

Double labelling fluorescence immunohistochemistry was employed to identify the cellular localisation of Igα_v, Igβ_3, Igβ_1 and CD44 receptors in the normal and LPS lesioned rat SN. The sections were stained for Igα_v, Igβ_3, Igβ_1 or CD44 and a marker of either neurones (Neuronal nuclei, NeuN), dopaminergic neurons (TH), astrocytes (GFAP), microglia (OX-42), activated microglia (OX-6) or macrophages (ED-1). The detailed method of this technique is described in Section 2.5.2.d. Briefly, SN sections were incubated with two different primary antibodies raised in different species (Table 2.5) overnight at 4˚C. On the next day, sections were incubated with a mixture of Alexa Fluor 594 (red) and Alexa Fluor 488 (green) secondary antibodies for one hour in a darkened environment. After three 30min washes with 0.1M PBS, coverslips were mounted, in the dark, onto polysine slides (Menzel-glaser) using vectashield hardset mounting medium with DAPI.

5.2.6 Data analysis

<u>TH</u>

The number of TH positive cells was manually counted from the whole SNpc (3 sections at the level of third nerve from each animal) in a blinded manner under magnification x20 using a Zeiss Axioskop microscope.

<u>Glial and macrophage markers</u>

The number of OX-6 and ED-1 positive cells was manually counted from 3 sections of the SN per rat, in a blinded manner. Immunopositive cells were counted from five random areas per section using a square grid (area= 0.25mm^2) under magnification x20.

<u>Receptors and GFAP</u>

Optical density of immuonostaining was measured for sections stained with GFAP, Igα_v, Igβ_3, Igβ_1 or CD44 relative to optical density of the background using ImageJ software. Images for this were taken using Olympus BX-61 microscope at magnification x1.25. Values are given in arbitrary units (AU).

5.2.7 Statistical Analysis

Data are expressed as mean±SEM of experimental treatment groups (n=3-8). Data were analysed by two-way ANOVA followed by Newman Keuls test. $P<0.05$ was considered significant. GraphPad Prism 5 software was used to analyse the data.

5.3 Results

5.3.1 Expression of Igα$_v$, Igβ$_3$, Igβ$_1$ and CD44 receptors in the normal rat brain SN

Igα$_v$, Igβ$_3$ and CD44 receptors were expressed in the SNpc and SN pars reticulata (SNpr) (Figure 5.2). Staining of Igα$_v$, Igβ$_3$ and CD44 receptors appeared in the cell bodies located mainly in the SNpc and some in the SNpr. Igβ$_3$ stained smaller areas of cell bodies than Igα$_v$ and CD44. Igβ$_1$ receptor was expressed to a lesser extent in the SN and not consistently through the SN (Figure 5.2c). Staining of Igβ$_1$ receptor was also located in the cell bodies. Antibodies for all receptors also faintly stained cell processes (Figure 5.2).

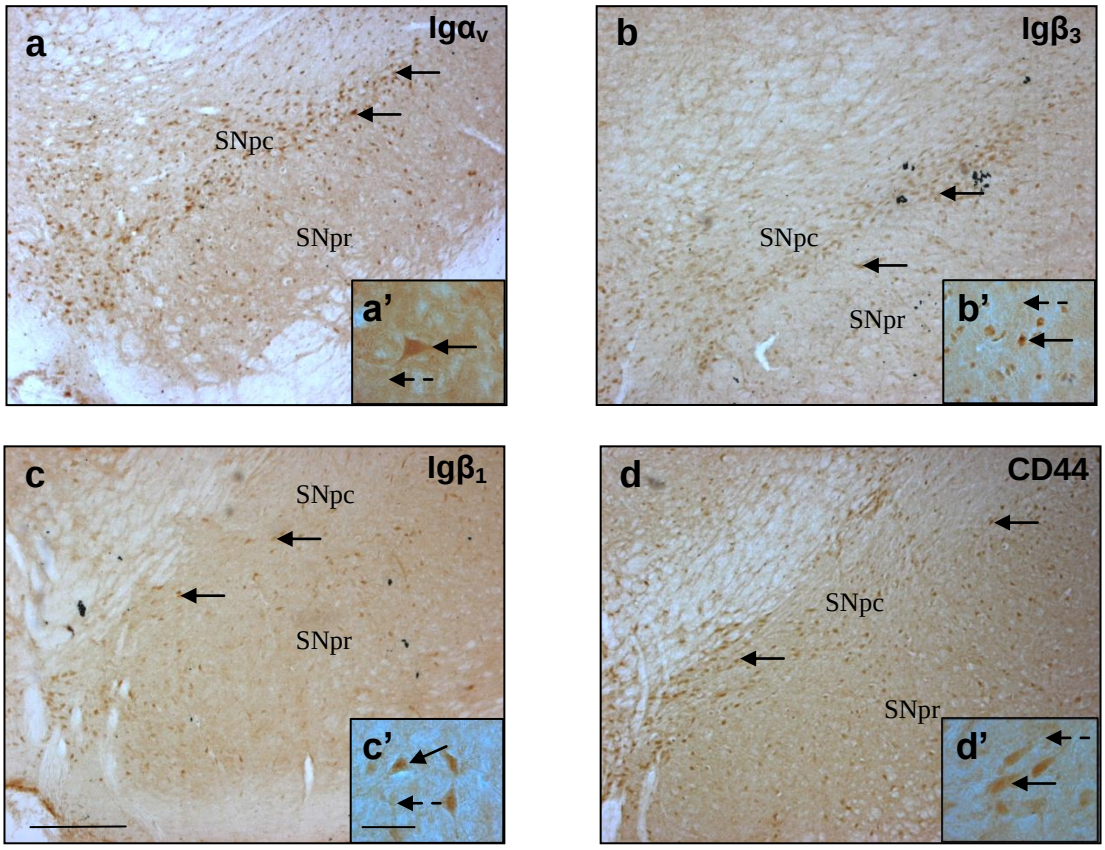

Figure 5.2 Peroxidase immunostaining of Igα$_v$, Igβ$_3$ and Igβ$_1$ and CD44 receptors in the rat SN.

Representative photomicrographs showing (a) Igα$_v$ positive cells, (b) Igβ$_3$ positive cells, (c) Igβ$_1$ positive cells and (d) CD44 positive cells in the SN. Magnification x5, → receptor positive cells, scale bar = 1000µm (c) and is representative of all photomicrographs. Inserts (a'-d'), magnification x40, scale bar = 50 µm (c') and is representative of all inserts. -→ receptor positive processes.

5.3.2 Cellular localisation of Igα_v, Igβ_3, Igβ_1 and CD44 receptors in the SN

Igα_v positive staining appeared in cell nuclei with fainter staining in cell bodies (Figures 5.2-5.3). Igβ_3 positive staining appeared mainly in the cell nucleus with fainter staining over some of the cell body (Figures 5.2-5.3). CD44 positive staining appeared over the whole cell body (Figures 5.2-5.3). Igβ_1 was not detected in the SN by immunofluorescence histochemistry.

5.3.2.a Co-localisation of Igα_v, Igβ_3 and CD44 receptors with neurones of the SN

Igα_v, Igβ_3 and CD44 receptors were expressed in almost all neurons of the SN as identified by NeuN co-staining (Figure 5.3). Igα_v, Igβ_3 and CD44 receptors were also present in cells that were not NeuN positive suggesting that they are also expressed in non neuronal cells.

5.3.2.b Co-localisation of Igα_v, Igβ_3 and CD44 receptors with Dopaminergic neurons of the SN

Igα_v, Igβ_3 and CD44 receptors were present in TH positive neurons of the SN as shown by co-localisation of immunofluorescence staining of these receptors and TH immunoreactivity (Figure 5.4). However, Igα_v, Igβ_3 and CD44 receptors were also expressed on other cells that were not TH positive.

5.3.2.c Co-localisation of Igα_v, Igβ_3 and CD44 receptors with glial cells of the SN

None of the receptor positive cells co-localised with GFAP positive astrocytes, although receptor positive cells were located in very close proximity to GFAP positive cells (Figure 5.5). Co-localisation with microglial cells could not be investigated as OX-42 signal for resting microglia could not be detected using the immunofluorescence technique.

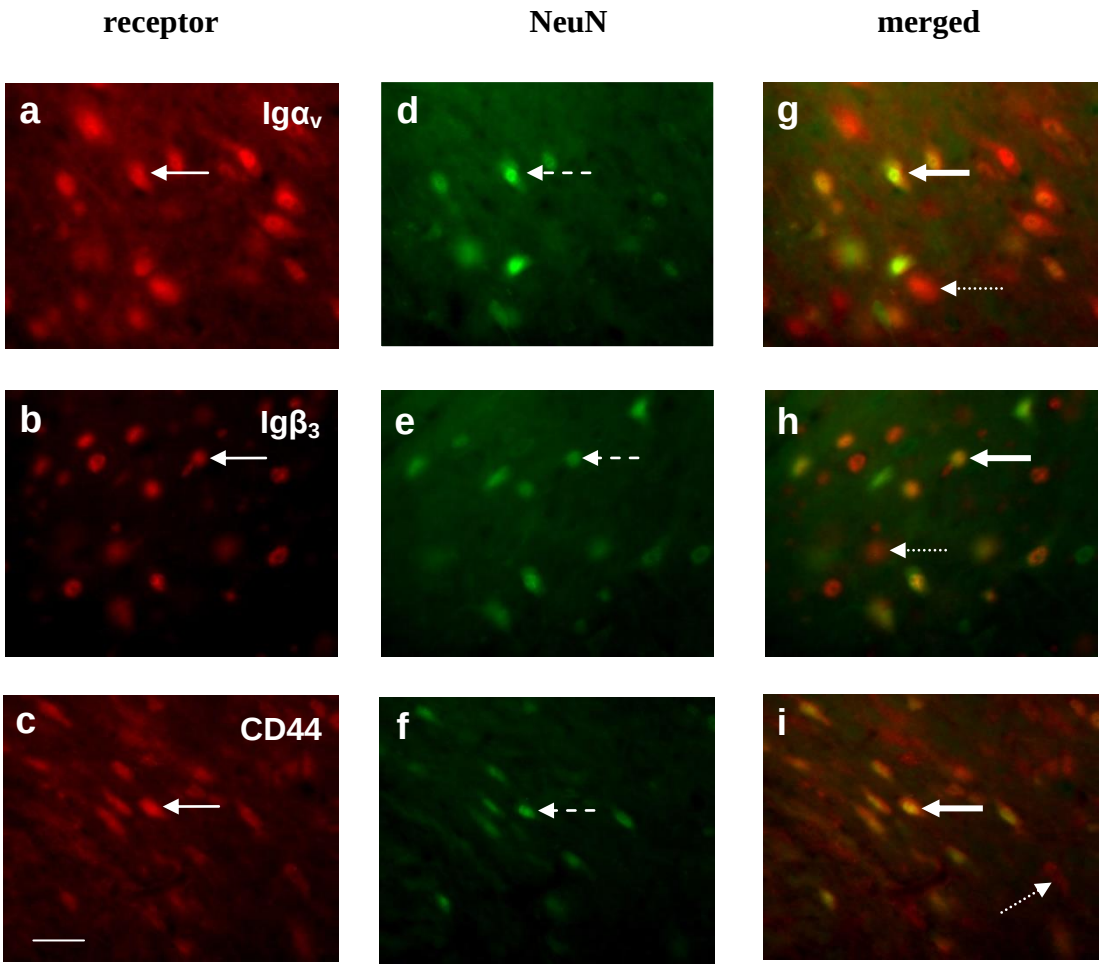

Figure 5.3 Immunofluorescence staining in SN sections showing co-localisation of NeuN positive neurons with Igα$_v$, Igβ$_3$ and CD44 receptors.

Representative photomicrographs showing Igα$_v$, Igβ$_3$ or CD44 receptor positive cells (a-c; red, ⟶), NeuN positive cells (d-f; green, - - ▶), cells expressing both receptor and NeuN (g-i, ⟶) and cells expressing receptor but not NeuN (g-i; red, ⋯▶) in the SN. Magnification x40, scale bar = 100μm and is representative of all images.

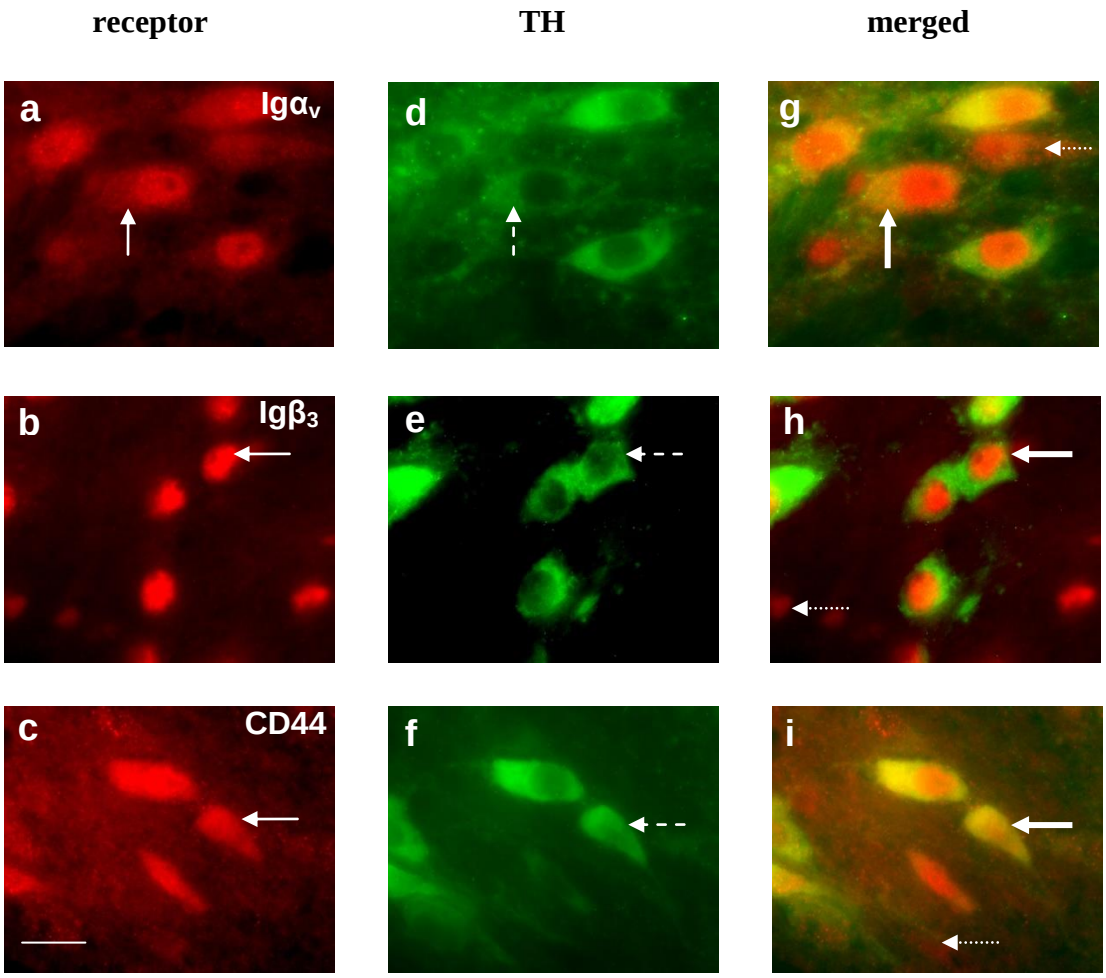

Figure 5.4 Immunofluorescence staining in SN sections showing co-localisation of TH positive neurons with Igα$_v$, Igβ$_3$ and CD44 receptors.

Representative photomicrographs showing Igα$_v$, Igβ$_3$ or CD44 receptor positive cells (a-c; red, →), TH positive cells (d-f; green, - -►), cells expressing both receptor and TH (g-i; ➡) and cells expressing receptor but not TH (g-i; red, ·····►) in the SN. Magnification x100, scale bar = 50µm and is representative of all images.

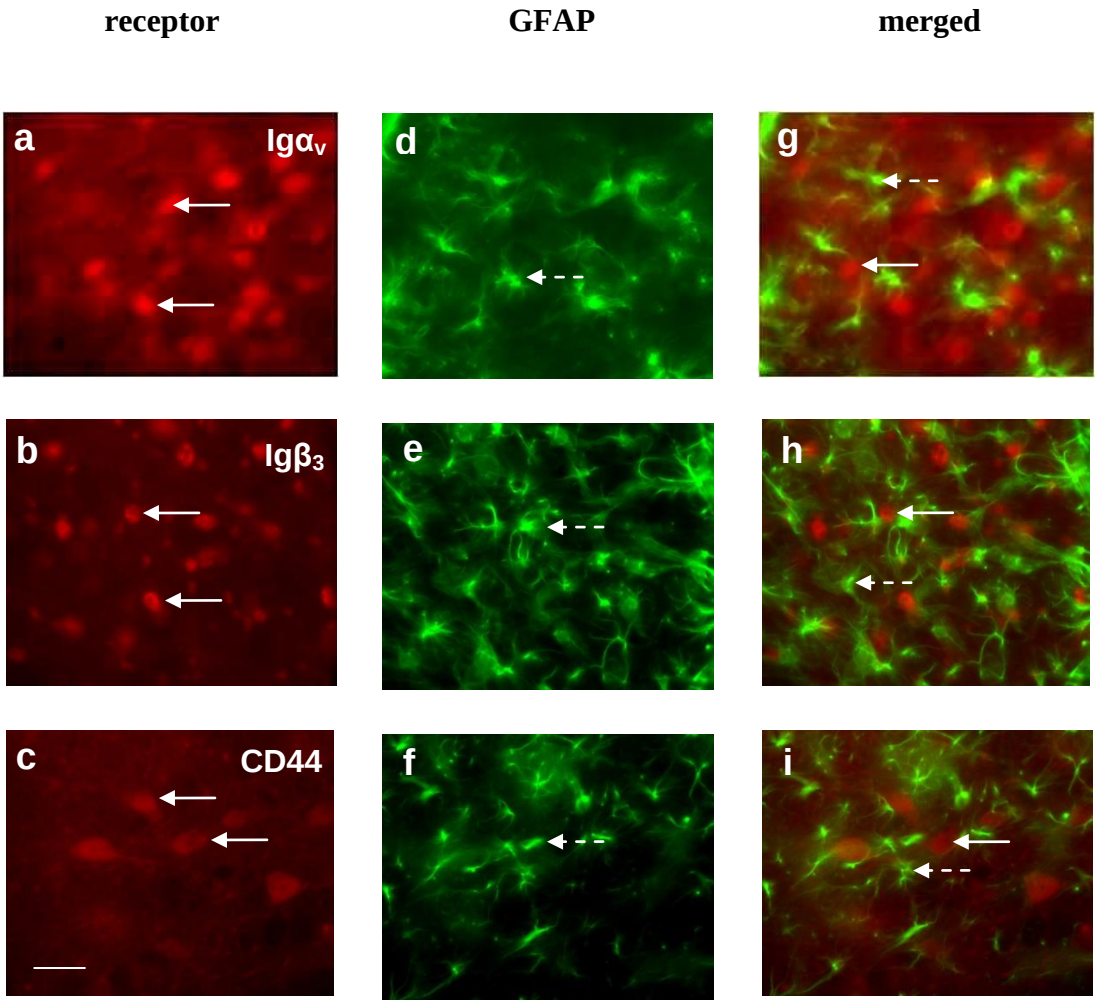

Figure 5.5 Immunofluorescence staining in SN sections showing expression of GFAP and Igα$_v$, Igβ$_3$ and CD44 receptors.

Representative photomicrographs showing Igα$_v$, Igβ$_3$ or CD44 receptor positive cells (a-c; red, ⟶) and GFAP positive cells (d-f; green, - -▸) in the SN. No co-localisation was found between GFAP and receptors (g-i). Magnification x40, scale bar = 100µm and is representative of all images.

5.3.3 Establishing the rat LPS model

5.3.3.a Confirmation of TH positive cell loss and presence of gliosis following supra-nigral LPS injection

In order to investigate the effect of supra-nigral LPS injection on dopaminergic neurons and inflammatory cells, rats were supra-nigrally lesioned with LPS (10μg) and sacrificed after 1, 3 or 7 days.

As expected, LPS injection produced a significant loss (70%) of dopaminergic neurons after 24hrs compared to contralateral side, which remained until one week after surgery (Figure 5.6). Immunohistochemical staining confirmed the reactive microgliosis produced by LPS (Figure 5.7). Evidence of a reactive microgliosis is demonstrated by presence of activated (OX-6 positive) microglial cells on the lesioned but not the intact side (Figure5.7).

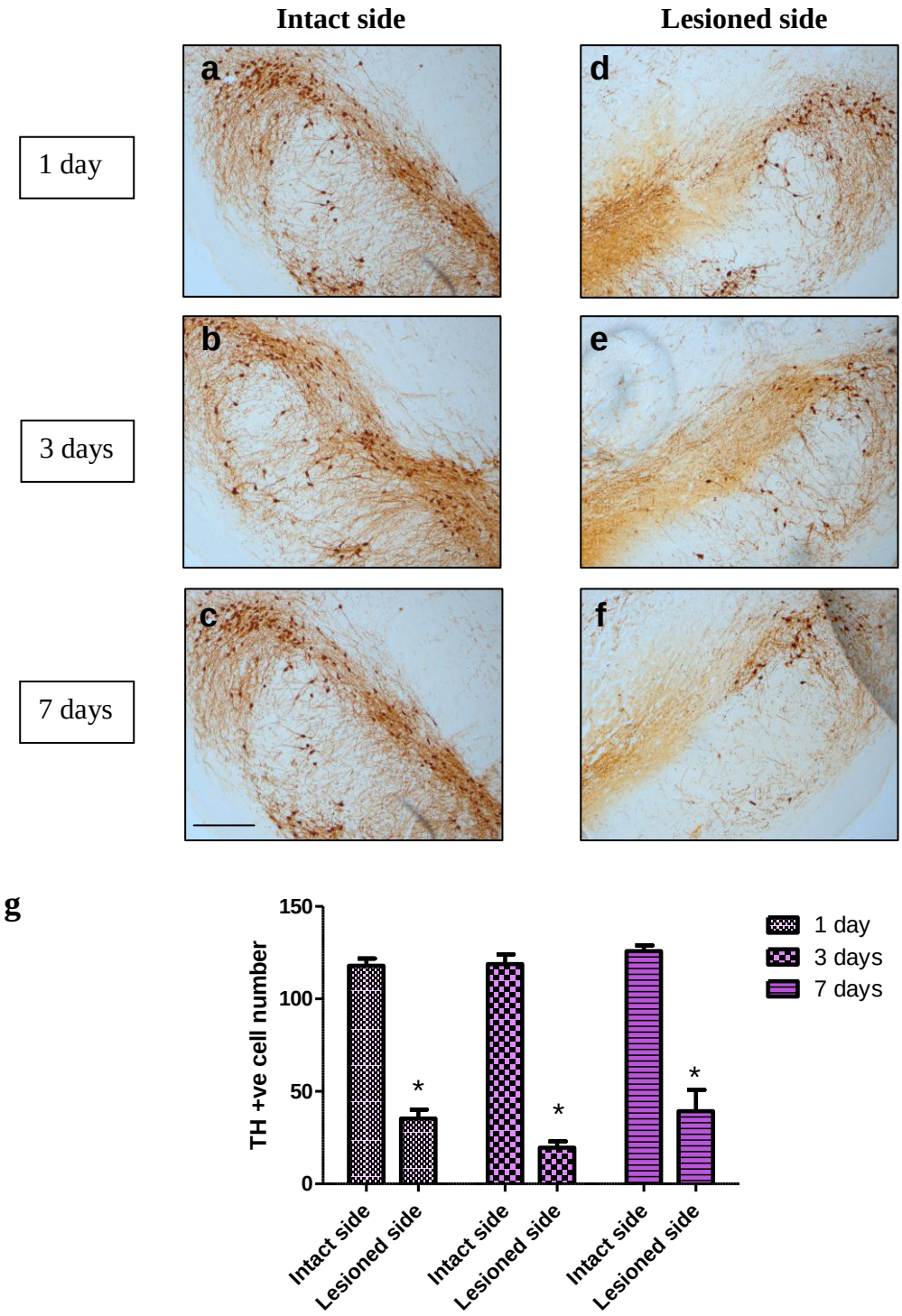

Figure 5.6 Dopaminergic cell loss following LPS lesioning.

Rats (n=6 per group) were lesioned with LPS (10µg) and culled after 1, 3 or 7 days post surgery. (a-f) Representative photomicrographs showing TH immunoreactivity in intact (a-e) and lesioned (b-f) sides of the SN at the different time points. Magnification x5, scale bar = 1000µm and is representative of all images. (g) The number of the remaining TH positive cells in the SN was counted. Data are expressed as mean ±SEM. *P<0.001 (two-way ANOVA followed by Newman Keuls test).

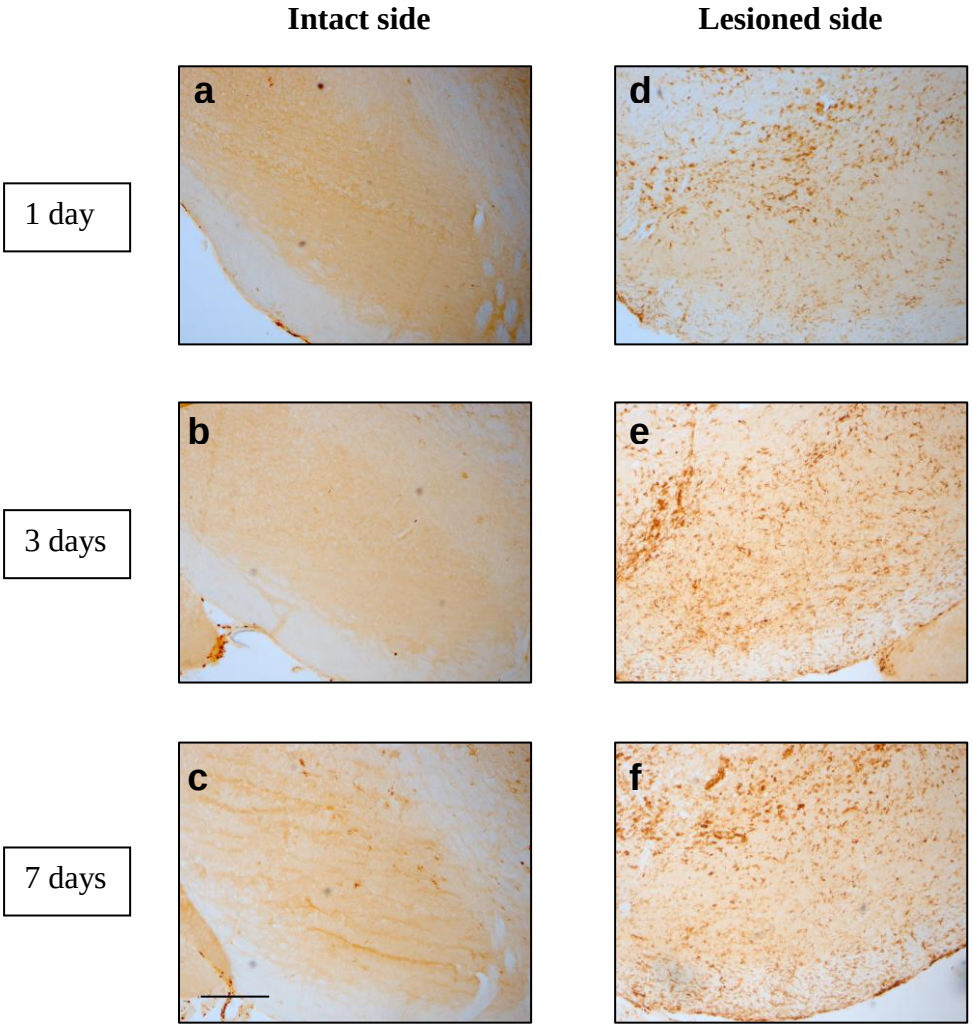

Figure 5.7 Microglioial activation in the SN following LPS lesioning.

Rats (n=6 per group) were lesioned with LPS (10µg) and culled after 1, 3 or 7 days. (a-f) Representative photomicrographs of OX-6 staining in intact and lesioned sides of SN sections (a) 1 day, (b) 3 days and (c) 7 days post LPS injection. Magnification x5, scale bar = 1000µm and is representative of all images.

5.3.4 Supra-nigral LPS injection induces up-regulation of integrin and CD44 receptors in the SN

In order to establish the effect of LPS on the expression of $Ig\alpha_v$, $Ig\beta_3$, $Ig\beta_1$ and CD44 receptors in SN, sections from rats supra-nigrally lesioned with LPS (10μg) and culled at three different time points (1 day, 3 days or 7 days) were stained for $Ig\alpha_v$, $Ig\beta_3$, $Ig\beta_1$ or CD44 by immunoperoxidase histochemistry and the degree of expression was represented by OD measurement.

LPS lesion produced an increase in the expression of $Ig\alpha_v$ (significant effect of lesion, $P<0.05$ two-way ANOVA), but the OD in the lesioned side of SN was not significantly different compared to the contralateral side at individual time points (Newman Keuls test) (Figures 5., 5.12). LPS injection induced a significant increase in the expression of $Ig\beta_3$ receptor in the ipsilateral side compared to the contralateral side at all time points and was highest at 7 days post injection (Figures 5.9, 5.12). LPS injection also significantly increased $Ig\beta_1$ expression in the ipsilateral side compared to the contralateral side at all time points but the increase of $Ig\beta_1$ expression was highest at day 7 post lesioning (Figures 5.10, 5.12). Expression of CD44 receptor was increased in the ipsilateral side compared to the contralateral side after LPS challenge in the SN at all time points (Figures 5.11, 5.12). Examining the sections at higher magnifications reveals that, for all receptors, both the intensity of staining of cell bodies and processes is higher after LPS lesioning (Figures 5.8-5.12).

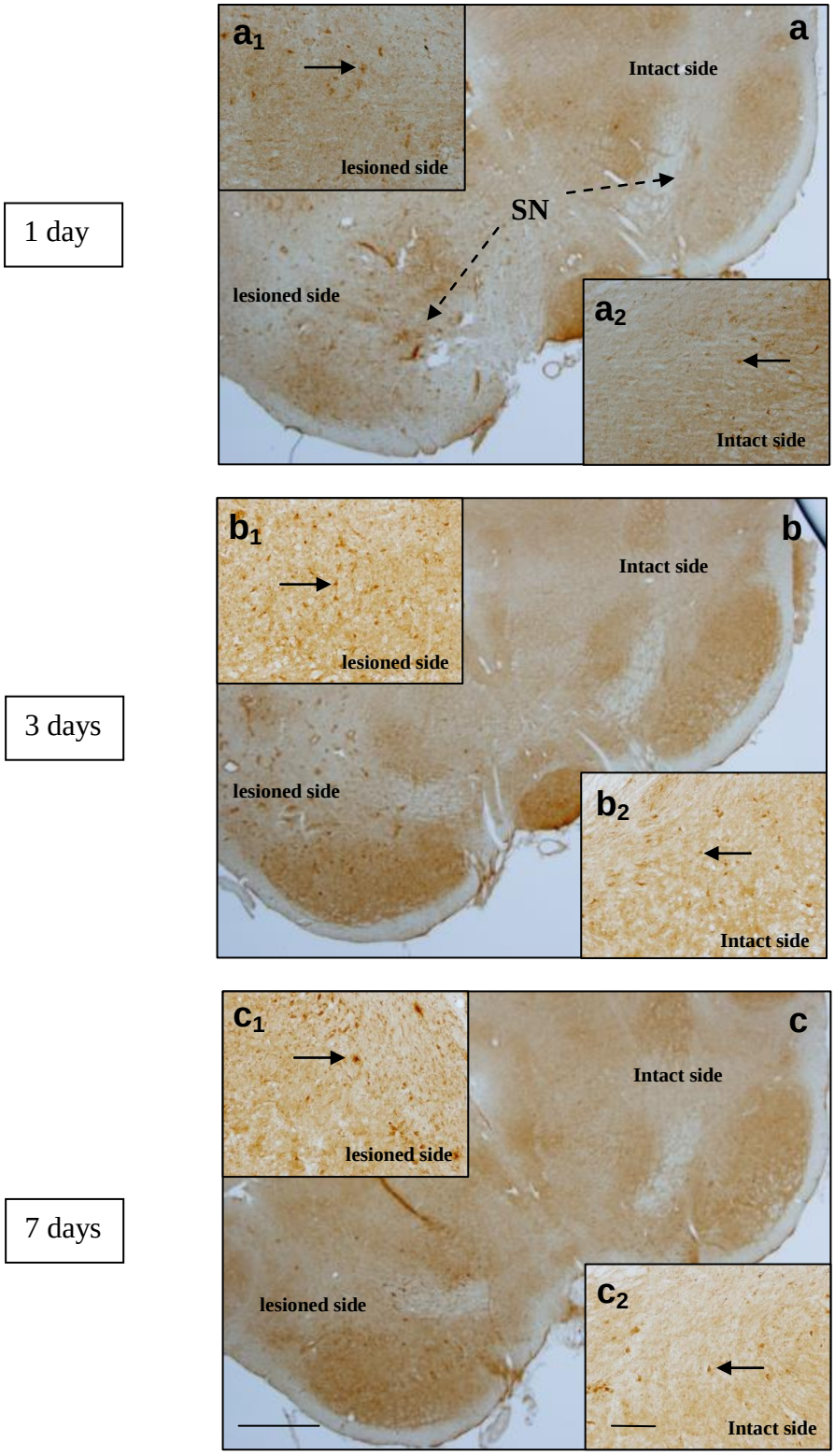

Figure 5.8 The effect of LPS on the expression of Igα$_v$ receptor in SN.

Rats were lesioned with LPS (10µg) and culled after 1 day, 3 days or 7 days post lesioning. Representative photomicrographs of Igα$_v$ staining in intact and lesioned sides of SN sections (a) 1 day, (b) 3 days and (c) 7 days post LPS injection. → Igα$_v$ positive cell. Magnification x1.25, scale bar = 3000µm and is representative of all images. Insets, magnification x10, scale bar (c_2) = 500µm and is representative of all insets.

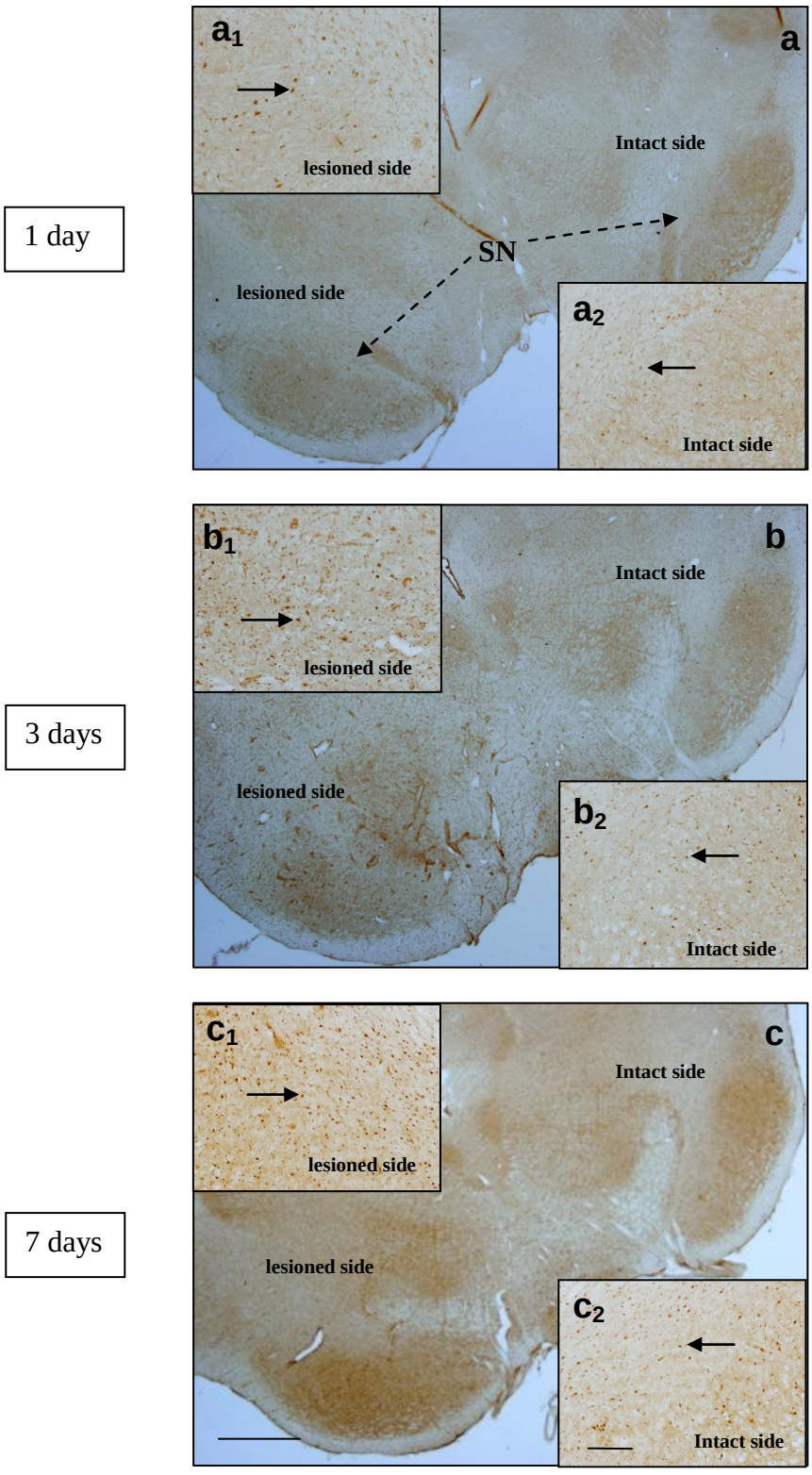

Figure 5.9 The effect of LPS on the expression of Igβ$_3$ receptor in SN

Rats were lesioned with LPS (10µg) and culled after 1 day, 3 days or 7 days post lesioning. Representative photomicrographs of Igβ$_3$ staining in intact and lesioned sides of SN sections (a) 1 day, (b) 3 days and (c) 7 days post LPS injection. → Igβ$_3$ positive cell. Magnification x1.25, scale bar = 3000µm and is representative of all images. Insets, magnification x10, scale bar (c_2) = 500µm and is representative of all insets.

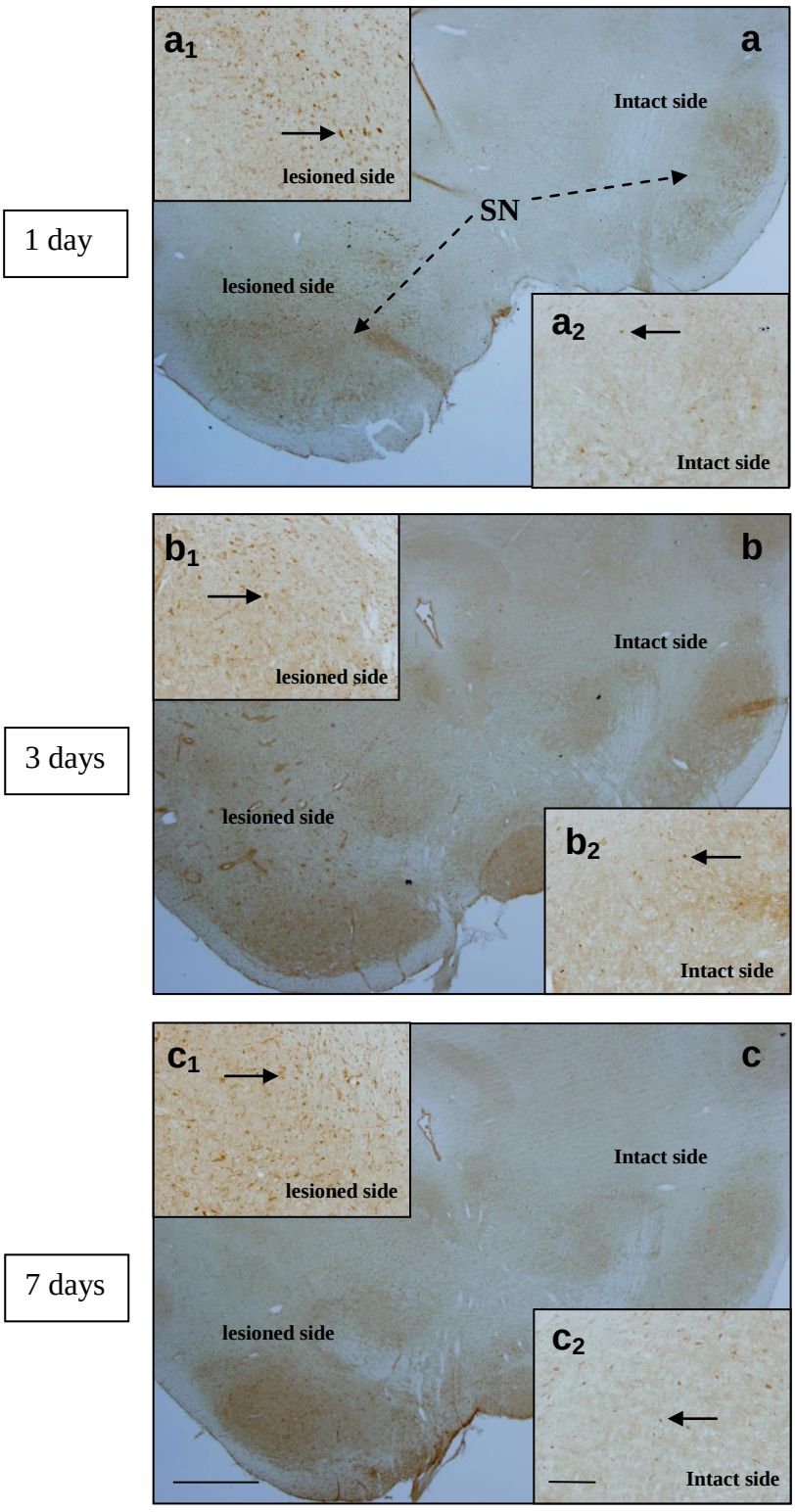

Figure 5.10 The effect of LPS on the expression of Igβ$_1$ receptor in SN

Rats were lesioned with LPS (10µg) and culled after 1 day, 3 days or 7 days post lesioning. Representative photomicrographs Igβ$_1$ staining in intact and lesioned sides of SN sections (a) 1 day, (b) 3 days and (c) 7 days post LPS injection. → Igβ$_1$ positive cell. Magnification x1.25, scale bar = 3000µm and is representative of all images. Insets, magnification x10, scale bar (c_2) = 500µm and is representative of all insets.

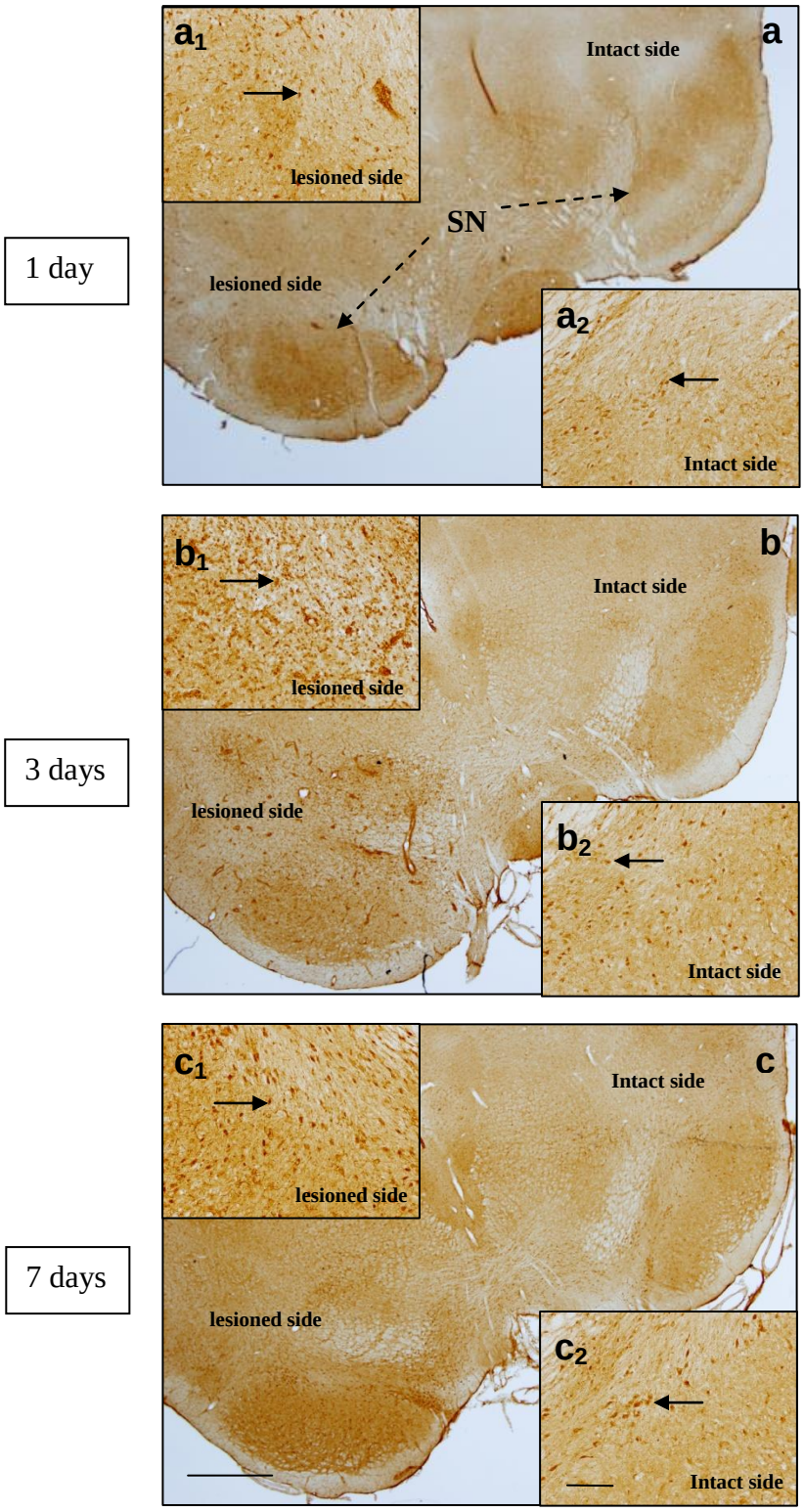

Figure 5.11 The effect of LPS on the expression of CD44 receptor in SN

Rats were lesioned with LPS (10µg) and culled after 1 day, 3 days or 7 days post lesioning. Representative photomicrographs of CD44 staining in intact and lesioned sides of SN sections (a) 1 day, (b) 3 days and (c) 7 days post LPS injection. → CD44 positive cell. Magnification x1.25, scale bar = 1000µm and is representative of all images. Insets, magnification x10, scale bar (c_2) = 500µm and is representative of all insets.

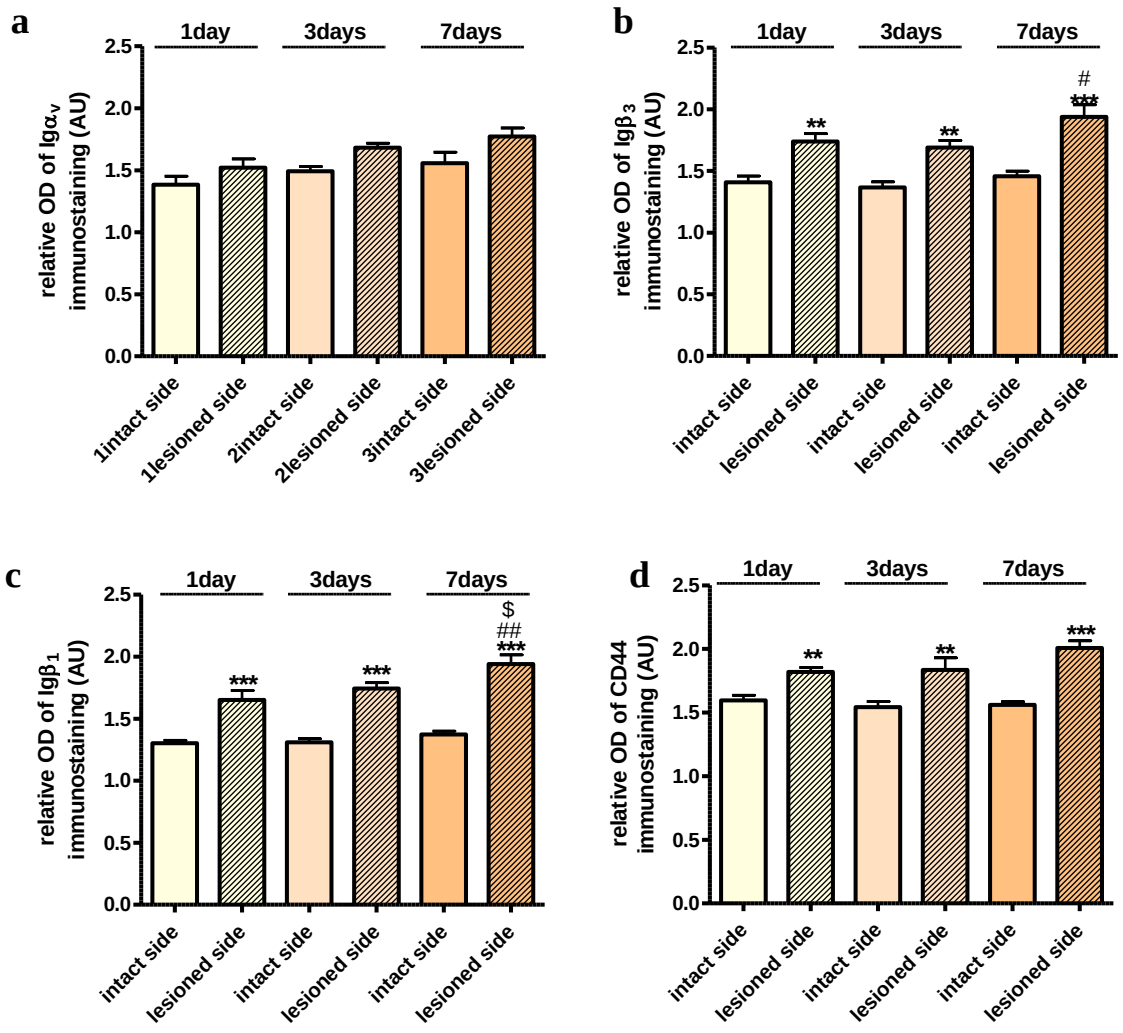

Figure 5.12 The effect of LPS on the expression of Igα$_v$, Igβ$_3$, Igβ$_1$ and CD44 receptors in SN

Rats (n=6 per group) were lesioned with LPS (10µg) and culled after 1, 3 or 7 days post lesioning. Optical density of receptor staining relative to background was measured for (a) Igα$_v$, (b) Igβ$_3$, (c) Igβ$_1$ and (d) CD44 immunostaining in the SN. Data are expressed as mean ±SEM. **P<0.01, ***P<0.001 compared to intact side; # P<0.05, ##P<0.01 compared to 1day lesioned side; $ P<0.05 compared to 3days lesioned side (two-way ANOVA followed by Newman Keuls test).

5.3.5 Integrin and CD44 receptors are expressed on glial cells following LPS challenge in the SN

In order to determine the effect of LPS on the cellular localisation of Igα_v, Igβ_3, Igβ_1 and CD44 receptors in SN, sections from rats lesioned supra-nigrally with LPS (10μg) and culled at three different time points (1 day, 3 days and 7 days) were immunostained for Igα_v, Igβ_3, Igβ_1 or CD44 and OX-6, ED-1 or GFAP.

Integrins α_v and β_3 mainly stained nuclei and cell bodies while CD44 stained cell bodies and processes (Figure 5.12-5.14). Double labelling immunofluorescence showed that Igα_v, Igβ_3, Igβ_1 and CD44 receptors were expressed on some but not all OX-6 positive activated microglia, GFAP positive astrocytes and ED-1 positive macrophages (Figures 5.13-5.15). There was no difference in the co-localisation of Igαv, Igβ3, Igβ1 and CD44 receptors with inflammatory cells at all time points.

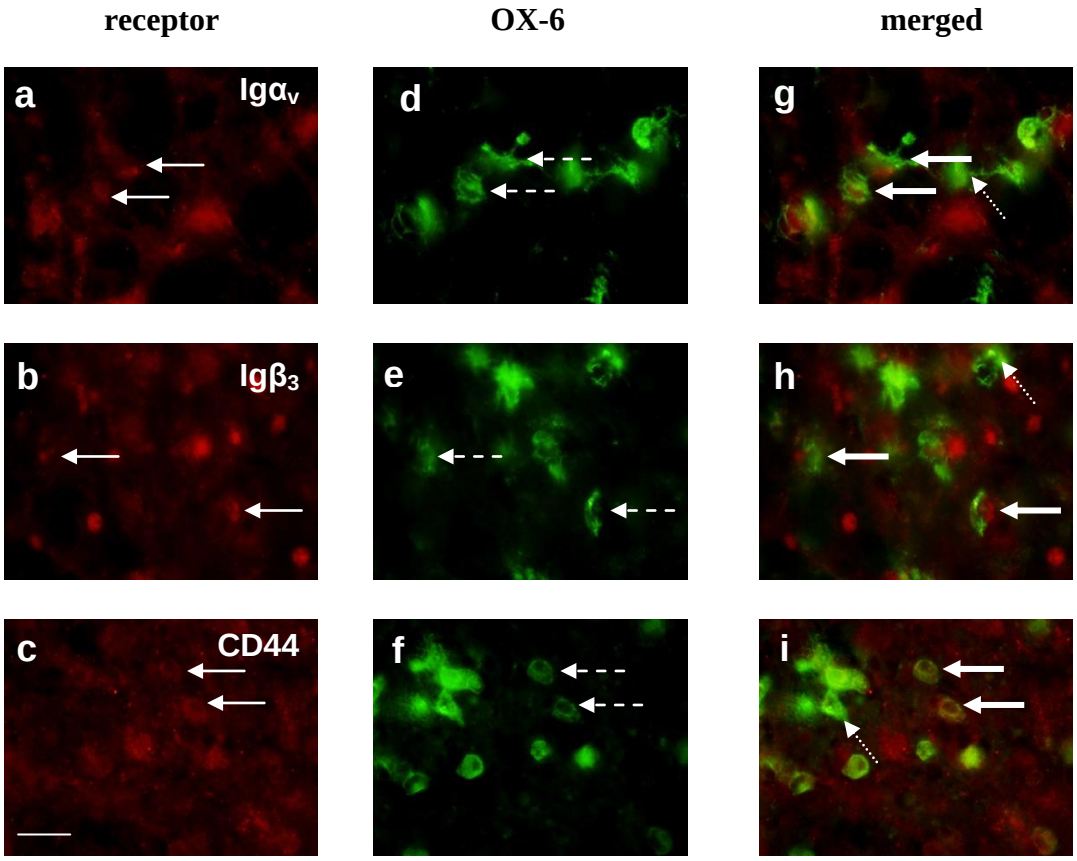

Figure 5.13 Immunofluorescence staining in SN sections showing expression of OX-6 positive microglia and Igα$_v$, Igβ$_3$ and CD44 receptors.

Representative photomicrographs showing Igα$_v$, Igβ$_3$ or CD44 receptor positive cells (a-c; red, →) , OX-6 positive cells (d-f; green, - - →), cells expressing both receptor and OX-6 (g-i; ➡) and cells expressing OX-6 but not the receptors (g-i, ⋯→) in LPS lesioned SN. Magnification x60, scale bar = 100µm and is representative of all images.

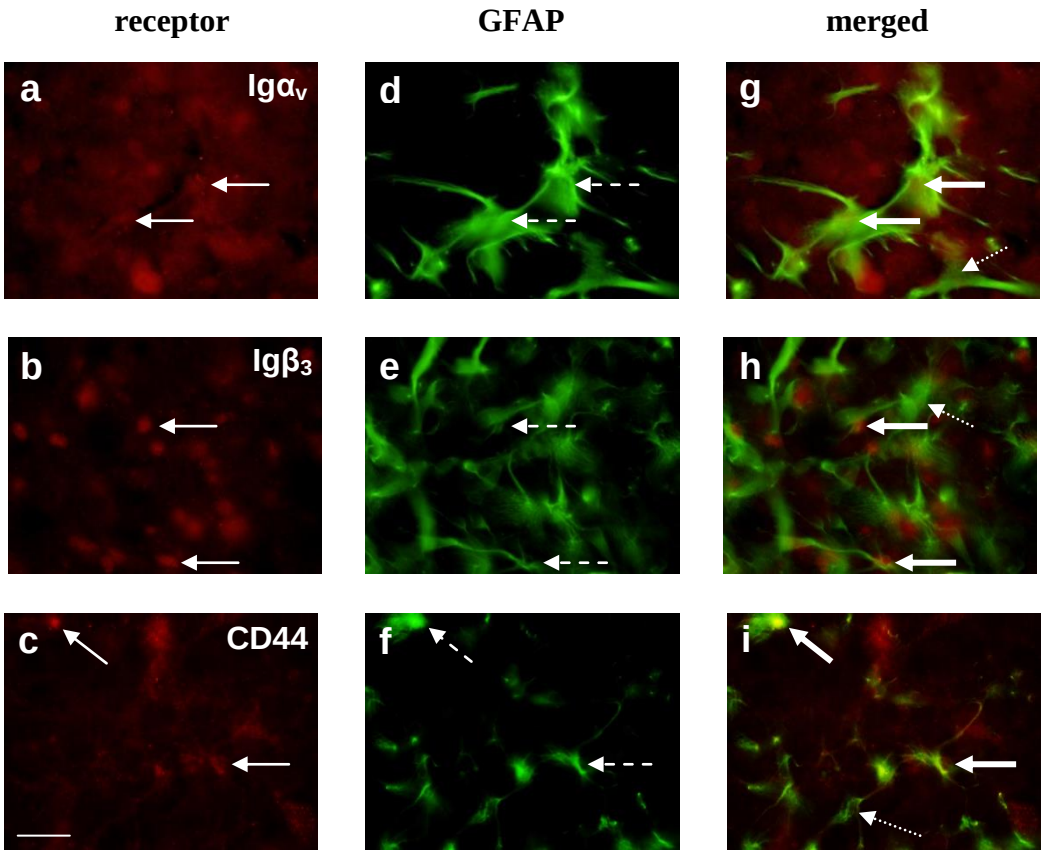

Figure 5.14 Immunofluorescence staining in SN sections showing expression of GFAP positive astrocytes and Igα$_v$, Igβ$_3$ and CD44 receptors.

Representative photomicrographs showing Igα$_v$, Igβ$_3$ or CD44 receptor positive cells (a-c; red, ⟶), GFAP positive cells (d-f; green, - -▸), cells expressing both receptor and GFAP (g-i, ➡) and cells expressing GFAP but not the receptors (g-i, ⋯▸) in LPS lesioned SN. Magnification x60, scale bar = 100µm and is representative of all images.

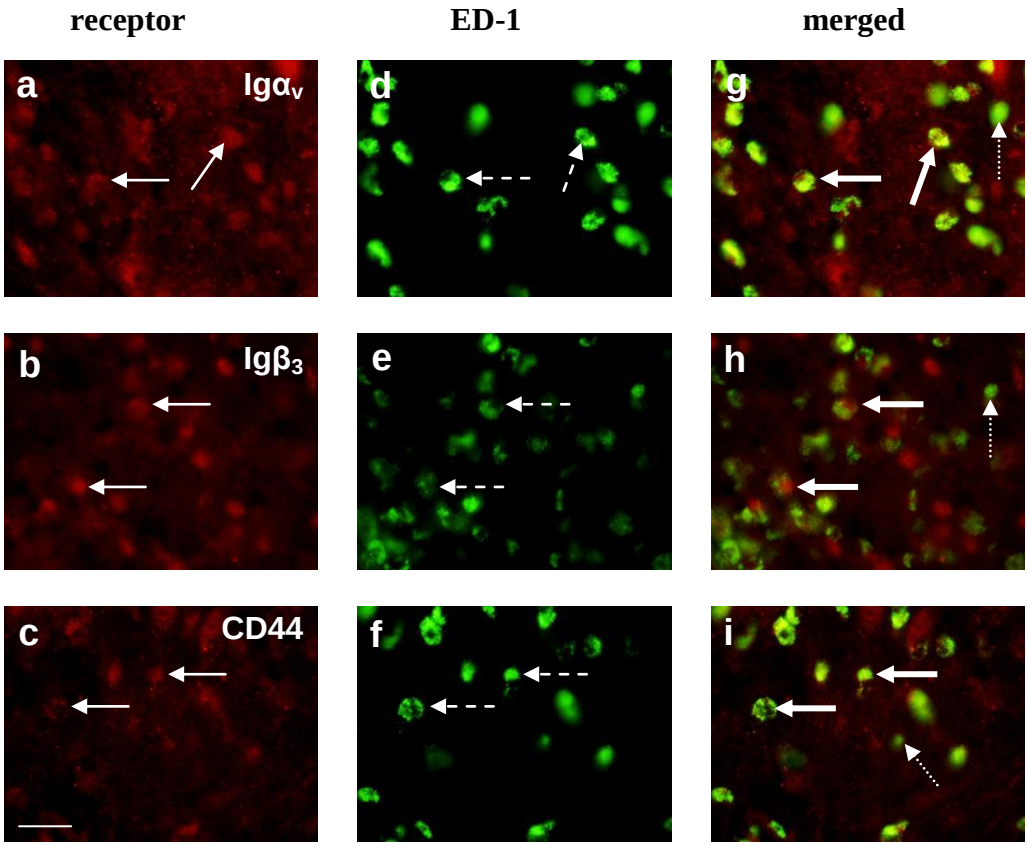

Figure 5.15 Immunofluorescence staining in SN sections showing expression of ED-1 positive macrophages and Igα$_v$, Igβ$_3$ and CD44 receptors.

Representative photomicrographs showing Igα$_v$, Igβ$_3$ or CD44 receptor positive cells (a-c; red, →), ED-1 positive cells (e-f; green, - -►) and cells expressing both receptor and ED-1 (g-i, ➡) and cells expressing ED-1 but not receptor (g-i, ·····►) in LPS lesioned SN. Magnification x60, scale bar = 100µm and is representative of all images.

5.3.6 The effect of OPN on nigral TH positive cell loss induced by LPS lesioning

In order to investigate whether OPN treatment in the SN is protective against LPS toxicity, recombinant rat OPN (100ng, 2µl) or vehicle (PBS, 2µl) were injected 2 or 24hrs into the SN before injecting LPS (10µg, 2µl) supra-nigrally.

No significant effects on the number of TH-positive cells were observed in sham-lesioned and vehicle-injected rats nor in sham-lesioned and OPN-injected rats (Figure 5.16). As expected, LPS produced a 50% (2hr study) and a 55% (24hr study) loss of TH positive cells in the lesioned SN compared to vehicle lesioned control (Figure 5.16). However, OPN injection into the SN 24hr before LPS lesioning significantly protected against TH-positive cell loss in the SN and the number of TH positive cells was not different to control (Figure 5.16-e). Injecting OPN only 2hrs before LPS lesioning provided similar protection to dopaminergic neurons (Figure 5.16-f).

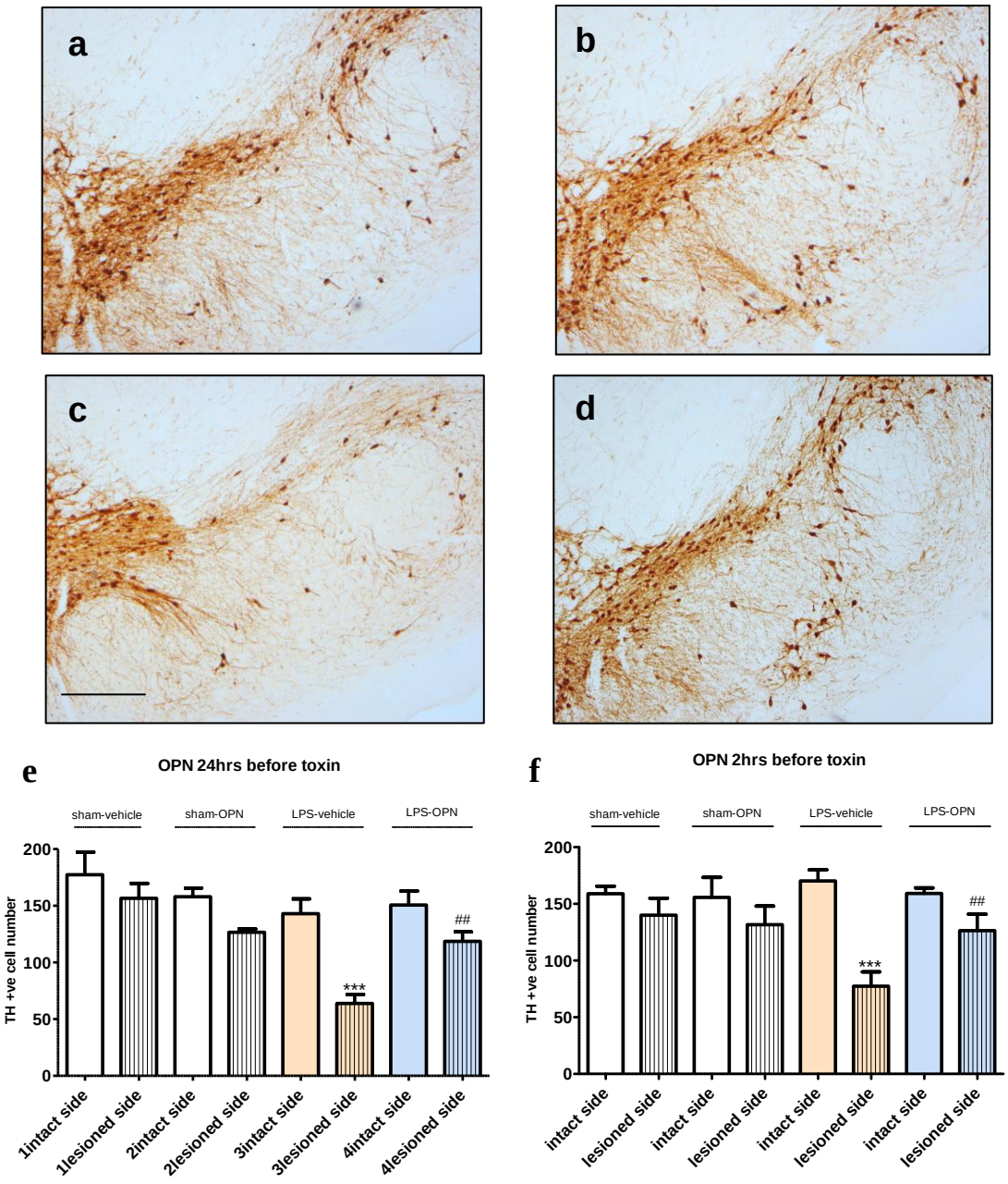

Figure 5.16 The effect of OPN on the number of TH –positive cells in SN of LPS or sham-lesioned rats.

(a-d) Representative photomicrographs of TH immunoreactivity in the lesioned side of SN in (a) sham-lesioned and vehicle-injected rats, (b) sham-lesioned and OPN-injected rats, (c) LPS-lesioned and vehicle injected rats, (d) LPS-lesioned and OPN-injected rats. Magnification x5, scale bar = 1000µm and is representative of all images. (e) Number of TH-positive cells in the lesioned and intact SN following OPN or vehicle injection 2hrs before LPS or vehicle lesioning (n=3-8). (f) Number of TH-positive cells in the ipsilateral and contralateral following OPN or vehicle injection 24hrs before LPS or vehicle lesioning (n=3-8). Data are expressed as mean ±SEM. ***P<0.001 compared to sham-lesioned and vehicle-injected, ## P<0.01 compared to LPS-lesioned and vehicle-injected rats (two-way ANOVA followed by Newman Keuls test). Rats were culled 1 week after LPS lesioning.

5.3.7 Effects of OPN on the inflammation produced by supra-nigral LPS injection

5.3.7.a The effect of OPN on microglial activation following LPS lesioning

In order to investigate the effect of OPN injection into the SN on the reactive gliosis produced by LPS lesion, SN sections from rats sham-lesioned and vehicle-injected, sham-lesioned and OPN-injected, LPS-lesioned and vehicle-injected or in rats LPS-lesioned and OPN-injected were stained for OX-6; a marker for activated microglial cells.

There was a small non-significant increase in the number of OX-6 positive microglia on the ipsilateral side of sham-lesioned and vehicle-injected rats and sham-lesioned and OPN-injected rats compared to the contralateral side (Figure 5.17). This was mainly located around the injection site (Figure 5.17). As expected, LPS produced a significant increase in the number of OX-6 positive microglia (24hr study, 150±10; 2hr study, 142±15) in the ipsilateral side compared to the ipsilateral side of sham-lesioned and vehicle injected control (24hr study, 6±2; 2hr study, 24±20). OX-6 positive cells displayed large cell bodies and shorter processes (Figure 5.17.c'). However, OPN-injected 24hrs prior to LPS lesioning produced a significant decrease (58%) in the number of activated microglial cells compared to LPS lesioned vehicle injected rats (Figure 5.17-e). When injected only 2hr before LPS lesioning, OPN also reduced the LPS-induced increase in the number of OX-6 positive microglia by (72%) (Figure 5.17-f). In OPN pre-treated SN, OX-6 positive cells appeared less activated with smaller cell bodies than LPS and vehicle injected rats (Figure 5.17.d').

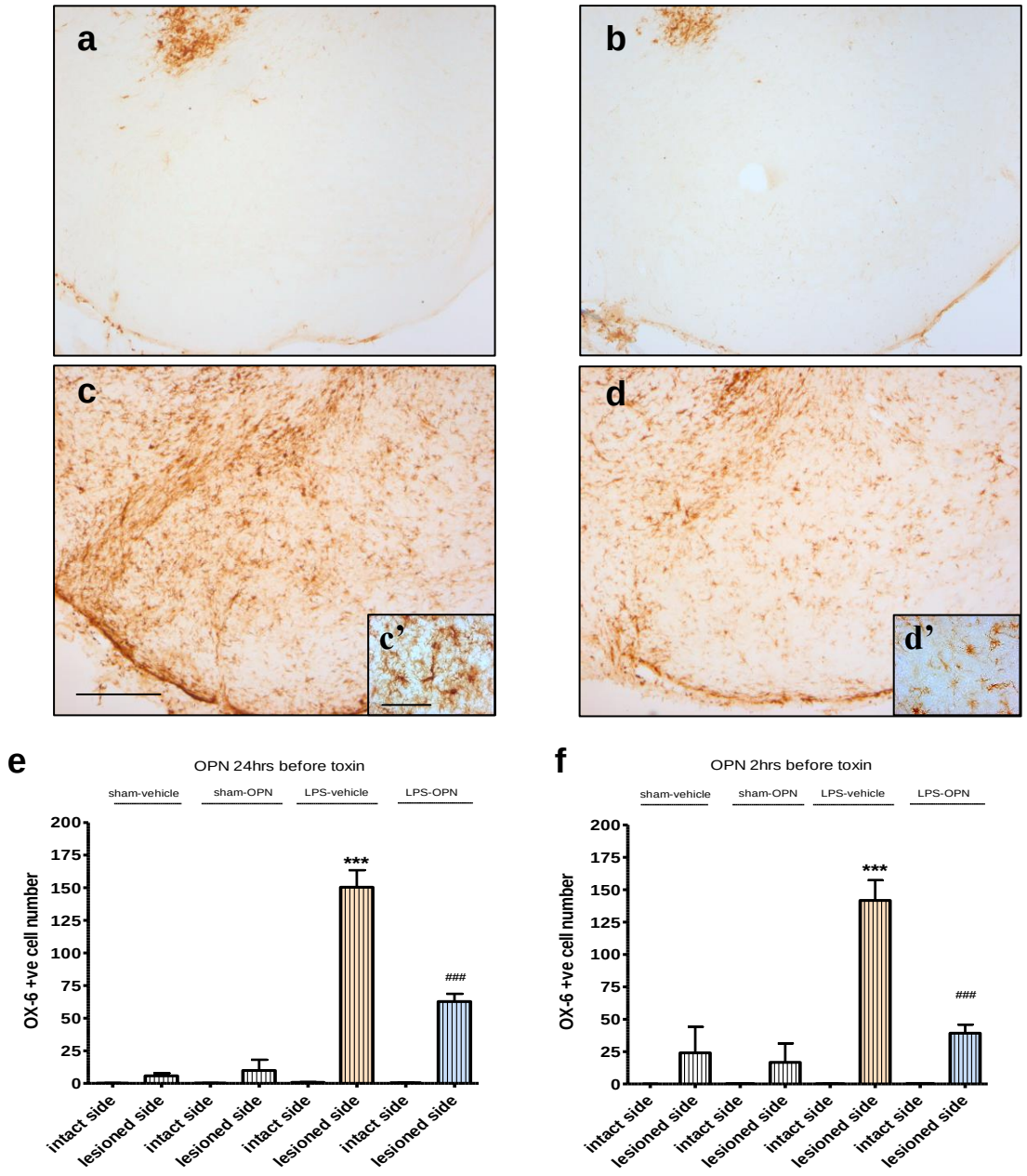

Figure 5.17 The effect of OPN on microglial activation produced by LPS in the SN.

(a-d) Representative photomicrographs of OX-6 immunoreactivity in the lesioned side of SN in (a) sham-lesioned and vehicle-injected rats, (b) sham-lesioned and OPN-injected rats, (c) LPS-lesioned and vehicle-injected rats, (d) LPS-lesioned and OPN-injected rats. Magnification x5, scale bar = 1000µm and is representative of all images. Insets (c',d'), magnification x40, scale bar = 50µm and is representative of both images. (e) Number of OX-6-positive cells in the lesioned and intact sides of SN following OPN or vehicle injection 2hrs before LPS or vehicle lesioning (n=3-8). (f) Number of TH-positive cells in the lesioned and intact sides of SN following OPN or vehicle injection 24hrs before LPS or vehicle lesioning (n=3-8). Data are expressed as mean ±SEM. ***P<0.001 compared to sham-lesioned vehicle-injected, ### P<0.001 compared to LPS-lesioned vehicle-injected rats (two-way ANOVA followed by Newman Keuls test). Rats were culled 1 week after LPS lesioning.

5.3.7.b The effect of OPN on the number of ED-1 positive macrophages following LPS lesioning

To study the effect of OPN injection into the SN on the macrophage infiltration produced by LPS lesion, SN section from the rats sham-lesioned and vehicle-injected, sham-lesioned and OPN-injected, LPS-lesioned and vehicle-injected or in rats LPS-lesioned and OPN-injected were stained for a marker of macrophages (ED-1).

There was a small non-significant increase in the number of ED-1 positive macrophages in the ipsilateral side of sham-lesioned and vehicle-treated rats and sham-lesioned and OPN-injected rats compared to the contralateral side (Figure 5.18). These macrophages showed small cell bodies with few processes (Figure 5.18.a'). As expected, LPS produced a significant increase in the number of ED-1 positive cells in the ipsilateral side compared to the sham-lesioned vehicle-injected control. In these rats, macrophages displayed larger cell bodies (Figure 5.18.c'). OPN-injected 2hrs or 24hrs before LPS lesioning did not have an effect on the increase in the number of activated macrophages induced by LPS lesioning (Figure 5.18). OPN injection 2hrs or 24hrs before LPS lesioning did not alter the morphology of activated macrophages.

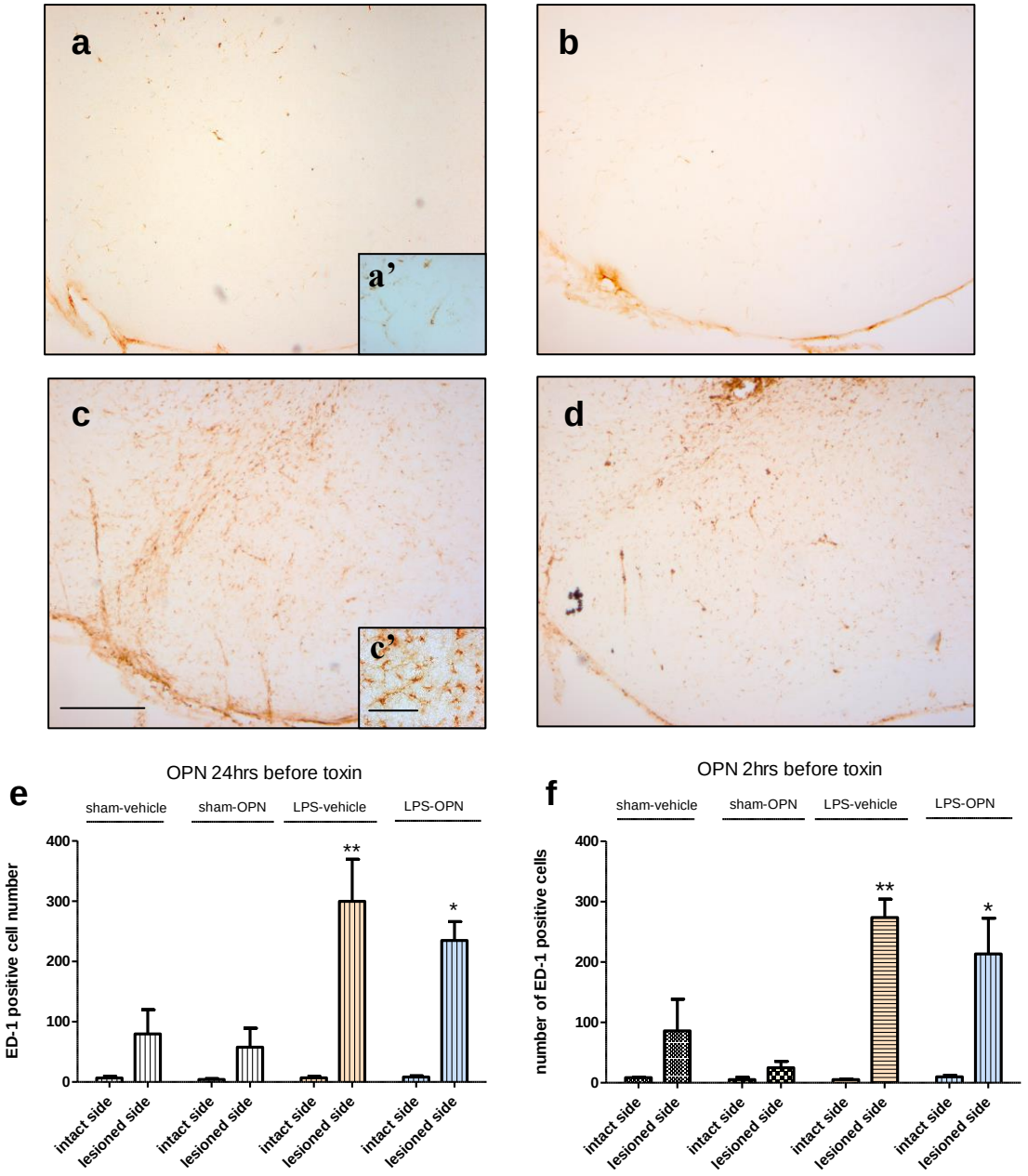

Figure 5.18 The effect of OPN and LPS on macrophages in the SN.

(a-d) Representative photomicrographs showing ED-1 immunoreactivity in the lesioned side of SN in (a) sham-lesioned and vehicle-injected rats, (b) sham-lesioned and OPN-injected rats, (c) LPS-lesioned and vehicle-injected rats, (d) LPS-lesioned and OPN-injected rats. Magnification x5, scale bar = 1000µm and is representative of all images. Insets (a',c'), magnification x40, scale bar = 50µm and is representative of both images. (e) Number of ED-1-positive cells in the lesioned and intact sides of SN following OPN or vehicle injection 2hrs before LPS or vehicle lesioning (n=3-8). (f) Number of ED-1-positive cells in the lesioned and intact sides of SN following OPN or vehicle injection 24hrs before LPS or vehicle lesioning (n=3-8). Data are expressed as mean ±SEM. *P<0.05 compared to sham-lesioned vehicle-injected, **P<0.01 compared to contralateral side (two-way ANOVA followed by Newman Keuls test). Rats were culled 1 week after LPS lesioning.

5.3.7.c The effect of OPN on the astrocytosis produced by LPS challenge

In order to investigate the effect of OPN injection into the SN on the reactive astrocytosis produced by LPS lesion, SN sections from rats sham-lesioned and vehicle-injected, sham-lesioned and OPN-injected, LPS-lesioned and vehicle-injected or in rats LPS-lesioned and OPN-injected were stained for a marker of astrocytes (GFAP) and optical density (OD) of staining was measured.

There was no change in the OD of GFAP staining in the ipsilateral side of sham-lesioned and vehicle-injected rats and in sham-lesioned and OPN-injected rats compared to the contralateral side (Figure 5.19). As expected, LPS induced a significant increase in the OD of GFAP immunostaining in the ipsilateral side compared to sham-lesioned vehicle-injected control. GFAP positive cells displayed hypertrophic cell bodies with thicker processes compared to control (Figure 5.19.c'). OPN injected 2hrs or 24hrs before LPS lesion did not have an effect on the increase in the OD of GFAP immunostaining induced by LPS lesioning nor on the morphology of astrocytes (Figure 5.19).

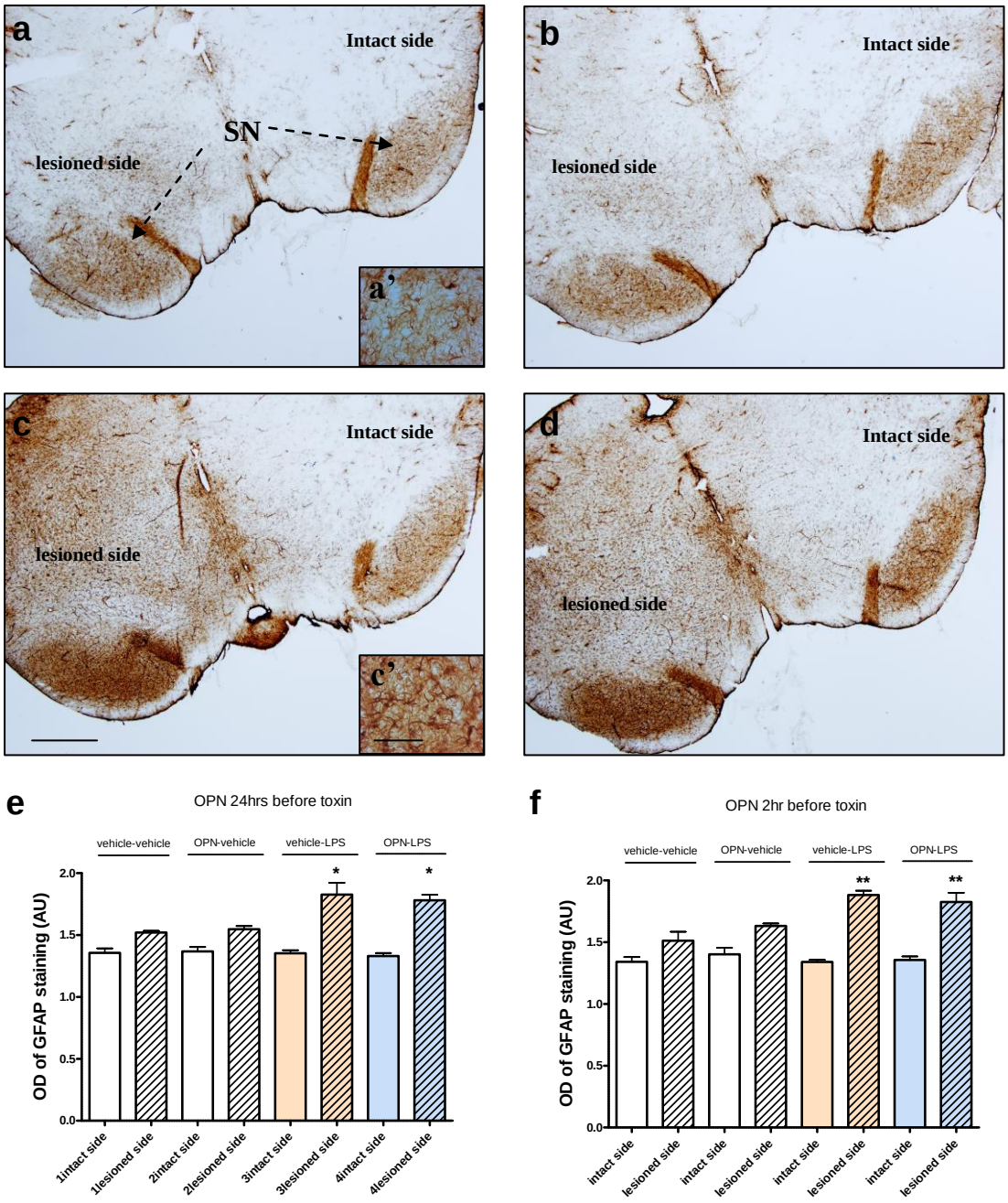

Figure 5.19 The effect of OPN on the astrocytosis produced by LPS in the SN.

(a-d) Representative photomicrographs showing GFAP immunoreactivity in the SN of (a) sham-lesioned and vehicle-injected rats, (b) sham-lesioned and OPN-injected rats, (c) LPS-lesioned and vehicle-injected rats, (d) LPS-lesioned and OPN-injected rats. Magnification x1.25, scale bar = 3000µm and is representative of all images. Insets (a',c') from lesioned side, magnification x40, scale bar = 50µm and is representative of both images. (e) OD of GFAP staining in the intact and lesioned side of the SN following OPN or vehicle injection 2hrs before LPS or vehicle lesioning (n=3-8). (f) OD of GFAP staining in the intact and lesioned side of the SN following OPN or vehicle injection 24hrs before LPS or vehicle lesioning (n=3-8). Data are expressed as mean ±SEM. *P<0.05 compared to sham-lesioned vehicle-injected, **P<0.01 compared to sham-lesioned vehicle-injected (two-way ANOVA followed by Newman Keuls test). Rats were culled 1 week after LPS lesioning.

5.4 Discussion

In these studies, it was hypothesized that OPN is protective against inflammation induced death of dopaminergic neurons and that this is mediated through an interaction with Igα_v, Igβ_3, Igβ_1 or CD44 receptors and an action on glial cells. Initial studies were undertaken to examine the SN for Igα_v, Igβ_3, Igβ_1 and CD44 receptors expression in normal and LPS-lesioned rats. This was followed by an investigation into the effect of nigral OPN injection on LPS-induced dopaminergic cell loss, and the potential involvement of glial cells in the protective effect.

5.4.1 Expression of Igα_v, Igβ_3, Igβ_1 and CD44 receptors in rat SN

Peroxidase immunohistochemistry investigation revealed that Igα_v, Igβ_3, Igβ_1 and CD44 receptors were all expressed in the SN. These findings confirm previous reports of the expression of mRNA of Igα_v and Igβ_1 in this brain region (Pinkstaff *et al.*, 1999). The same group could not detect a signal for β3 integrin but they describe unpublished Western blot data suggesting its presence in whole brain lysates (Pinkstaff *et al.*, 1999). Ig α_v mRNA and protein (Chao *et al.*, 2003) and the CD44 receptor (Fuxe *et al.*, 1996) were shown to be expressed in the rat SN. However, this is the first report of the expression of Igβ_3 and Igβ_1 protein in this brain region.

The cellular localisation of Igα_v, Igβ_3, Igβ_1 and CD44 receptors in the SN was investigated by double labelling immunofluorescence. Igα_v, Igβ_3 and CD44 were expressed on neurones as there was co-localisation between these receptors and the neuronal marker NeuN. In fact, Igα_v, Igβ_3 and CD44 receptors were found in all dopaminergic neurones of the SN as immunofluorescence results showed positive expression of Igα_v, Igβ_3 and CD44 receptors in TH-positive cells. The immunofluorescent signal for Igβ_1 was not different to negative control. This may be due to lower sensitivity of the imunnofluorescence technique compared to immunoperoxidase histochemistry.

Double labelling immunofluorescence showed no positive co-localisation of Igα_v, Igβ_3 and CD44 receptors with GFAP positive astrocytes in normal rat SN. However, there is a possibility that they co-localise with microglial cells but this

could not be investigated as resting microglial cells were not detected by immunofluorescence histochemistry due to lower sensitivity of this technique. A possible alternative for future studies would be to use in-situ hybridisation to label receptors followed by immunoperoxidase histochemistry to stain for microglial cells in order to examine co-localisation.

Presence of Igα_v, Igβ_3, Igβ_1 and CD44 receptors, involved in cell survival actions of OPN in other systems (Caers *et al.*, 2006; Lee *et al.*, 2007; Lin *et al.*, 2001; Scatena *et al.*, 1998), suggests that the SN may contain the mechanism for mediating pro-survival actions of OPN. However, it is not known whether expression of these receptors is altered in the LPS model of dopaminergic cell death, where OPN will be tested for neuroprotection.

5.4.2 The effects of LPS challenge in the SN

Supra-nigral LPS injection produced a significant loss of dopaminergic neurones from the SNpc compared to sham-lesioned control and this is in line with other reports (Herrera *et al.*, 2000; Iravani *et al.*, 2002; Iravani *et al.*, 2005). Previous studies have confirmed that LPS induces dopaminergic cell death rather than a reduced expression of TH protein (Gao *et al.*, 2002; Iravani *et al.*, 2002).

Supra-nigral LPS injection in the rat brain produced a significant increase in activated microglial cells, macrophages and astrocytes compared to sham-lesioned animals. This confirms previous evidence of the pro-inflammatory effects of LPS in the rat SN (Herrera *et al.*, 2000; Iravani *et al.*, 2002; Iravani *et al.*, 2005). LPS causes microglia and astrocyte activation (Castano *et al.*, 1998; Herrera *et al.*, 2000) and induces the expression of iNOS and an increase in the generation of NO and 3-NT from activated microglia (Arimoto *et al.*, 2003; Gayle *et al.*, 2002; Iravani *et al.*, 2002; Peng *et al.*, 2005). In addition, LPS leads to an increased expression of the pro-inflammatory cytokines TNF-α, IL-1α, IL-1β, and IL-6. This inflammatory process accompanying nigral cell degeneration is also seen in PD (Chapter1), but it is still not clear whether inflammation is a cause and/or an effect.

The expression of Igα_v, Igβ_3, Igβ_1 and CD44 receptors increased following LPS lesioning in the SN. Further investigation using double-immunofluorescence labelling showed evidence of expression of these receptors on some astrocytes,

activated microglia and macrophages present in the lesioned hemisphere of SN. This suggests that the up-regulation of Igα_v, Igβ_3 and CD44 receptors is likely to be due to the expression of these receptors on proliferating glial cells and infiltrating macrophages resulting from the LPS injection. This is the first time that the expression of these receptors was investigated in a PD model but up-regulation of integrins was observed in the temporal cortex in Alzheimer's disease (Akiyama *et al.*, 1991; Akiyama *et al.*, 1990), in brainstem and cerebellum in mice infused with LPS (Kloss *et al.*, 2001) and in multiple sclerosis lesions (Bo *et al.*, 1996). In these studies, integrin expression was increased in microglial cells. The CD44 receptor was also upregulated in Alzheimer's disease in GFAP positive astrocytes (Akiyama *et al.*, 1993), in a rat stroke model in microglia and macrophages (Wang *et al.*, 2001) and in a mouse model of multiple sclerosis in astrocytes (Haegel *et al.*, 1993). More importantly, CD44 expression was elevated in the SN of MPTP treated mice where it co-localised with infiltrating lymphocytes (Kurkowska-Jastrzebska *et al.*, 1999). The results obtained in the current study in addition to evidence from previous investigations indicate a possible role for integrin and CD44 receptors in the regulation of inflammation mediated neurodegeneration. This, however, requires further detailed investigation as it may be relevant to neurodegenerative diseases, where there is evidence of inflammatory involvement, such as PD. Integrin and CD44 receptors are known to bind many ligands and mediate various actions and may be mediating pro or anti-inflammatory effects. In support of this concept, Laminin and fibronectin (both integrin ligands) exert opposite effects on the activation state of cultured microglia from the cerebral cortex and striatum (Chamak *et al.*, 1991). Therefore OPN may have a role in regulating the LPS induced activation of inflammatory cells through effects on integrin or CD44 receptors.

5.4.3 The effects of OPN on LPS induced pathological changes

This is the first report of the neuroprotective effect of OPN against LPS induced loss of dopaminergic neurons in the SNpc. OPN injected into the SNpc 24hr or 2hr before supra-nigral LPS injection prevented dopaminergic neurone loss induced by LPS. This is in line with a recent study showing that a 15-mer peptide fragment of OPN containing the RGD binding domain is also protective in LPS and 6-OHDA lesioned rats (Iczkiewicz *et al.*, 2010). In the same study, intra-nigral injection of an anti-OPN antibody induced a significant loss of dopaminergic neurons

in the SN (Iczkiewicz *et al.*, 2010). In light of the evidence, OPN seems to be endogenously protective to dopaminergic neurons and when administered exogenously, provides protection against toxin induced cell death.

OPN caused a significant reduction in the LPS induced microglial activation but did not affect the increase of astrocytes or macrophages. This suggests that OPN may have protected dopaminergic neurons via preventing and/or attenuating the LPS induced microgliosis. This suggestion is supported by studies in models of stroke and ischemic neurodegeneration where OPN was found to be protective, this was accompanied by anti-inflammatory actions suggesting that it may protect neurons through reduction in inflammation induced cell death. In support of this concept, OPN KO mice displayed higher microglial activation and inflammatory gene expression in addition to higher secondary thalamic degeneration after cerebral artery occlusion compared to wild type animals (Schroeter *et al.*, 2006). In addition, following transient forebrain ischemia in rat, OPN was up-regulated in microglia and astrocytes but was also secreted into the extracellular space by astrocytes suggesting a role in tissue repair (Choi *et al.*, 2007). However, there are contradictory findings in multiple sclerosis and its animal model experimental autoimmune encephalomyelitis, where OPN is reported to be up-regulated mainly in microglia and macrophages and to increase survival and proliferation of T-cells resulting in a harmful pro-inflammatory effect (Chabas *et al.*, 2001; Hur *et al.*, 2007; Jansson *et al.*, 2002; Kim *et al.*, 2004). This discrepancy, nonetheless, may be due to the difference in disease models as the former is an ischemia mediated neurodegeneration and the latter is a T-cell mediated autoimmune disease.

The underlying protective mechanism of OPN may be accomplished by reducing nitrative stress. OPN can reduce nitrative stress by reducing levels of NO and iNOS as it was shown to inhibit up-regulation of NO and to lower mRNA levels of iNOS in kidney cells stimulated with LPS (Hwang *et al.*, 1994a; Hwang *et al.*, 1994b) and to inhibit production of NO in human osteoarthritis-affected cartilage (Attur *et al.*, 2001). Indeed, evidence of the presence of NO and iNOS exists not only in PD (Hunot *et al.*, 1996) but also in the LPS lesioned rats (Iravani *et al.*, 2002). Inhibition of NO up-regulation may be related to the evident reduction of activated microglial cell numbers in rats injected with OPN before LPS lesioning in this study, since iNOS is mainly expressed in activated microglia (Arimoto *et al.*,

2003; Dehmer *et al.*, 2000; Iravani *et al.*, 2005). The lower levels of NO would in turn reduce the generation of harmful peroxynitrite which forms after interaction of NO with superoxide anion produced in activated microglia. This suggested anti-inflammatory mechanism is further supported by the up-regulation of mediators of OPN actions ($Ig\alpha_v$, $Ig\beta_3$, and CD44 receptors) on glial cells post LPS lesionin. Moreover, studies in VM culture showed that integrins protected dopaminergic cells via a glial dependant mechanism (Peluffo *et al.*, 2007).

A possible alternative explanation is that OPN may be acting directly on neurons which were shown here to express $Ig\alpha_v$, $Ig\beta_3$ and CD44. This may be mediated through inhibiting apoptosis since there is a large body of evidence of anti-apoptotic and pro-survival effects of OPN in different cell types including neurons (Chapter 1). This, however, requires further investigation. It is also possible that OPN works via induction of neurotrophic factor release from glial cells since in unpublished studies from these laboratories OPN induced GDNF and BDNF release in VM cultures (Broom, 2011, personal communication). It is not excluded that OPN acts *both* directly on dopaminergic neurones and indirectly through an effect on glial cells. The exact mechanism of neuroprotection by OPN in the LPS model is not yet clear and prompts further study, but it seems to be related to an effect on activated microglia. Although OPN was shown to be neuroprotective in LPS lesioned rats, extensive investigations are still required to establish the exact role of OPN in PD.

5.4.4 Conclusion

In conclusion, these data confirm the dopaminergic cell loss and gliosis induced by LPS and show for the first time the ability of OPN protein to protect against LPS-induced dopaminergic cell loss. This was mediated via attenuating the microgliosis produced by LPS probably via a more complex anti-inflammatory mechanism. This is supported by the finding that OPN receptors were expressed on glial cells following LPS insult in the SN. Other direct effects of OPN on dopaminergic neurons cannot be ruled out at this stage, especially since OPN receptors are also expressed on dopaminergic neurons. The increase in the expression of integrin and CD44 receptors following LPS lesion indicates a possible role for these receptors in PD but requires further investigation.

Chapter 6 General Discussion

6.1 Thesis hypothesis and Aims

Based on previous reports of the involvement of OPN in the regulation of inflammation and its pro-survival effects, it was suggested that OPN may be a potential neuroprotective agent in PD. As a consequence, it was hypothesized that OPN is protective against toxic insult when endogenously expressed or when dopaminergic cells are exposed to it exogenously. It was also hypothesised that protective effects of OPN are mediated through an action on glial cells and via $Ig\alpha_v$, $Ig\beta_3$, $Ig\beta_1$ and CD44 receptors. Therefore, the aims of the studies described in this thesis were to investigate the protective effects of exogenous and endogenous OPN and to study the role of glial cells and $Ig\alpha_v$, $Ig\beta_3$, $Ig\beta_1$ and CD44 receptors in its effects.

6.2 Summary of results

- Endogenous OPN expression in dopaminergic cell lines or transfection of cell lines to express OPN did not protect against toxin induced cell death, nor did pre-treatment with exogenous OPN.
- OPN protected dopaminergic neurons in primary VM culture against MPP^+ toxicity.
- Protection by OPN in VM culture was mediated via integrin receptors and it was accompanied by an increase in the number of microglia.
- OPN also protected dopaminergic neurons of the SN from LPS induced cell death and reduced the number of activated microglia following LPS challenge but did not affect macrophage or astrocyte numbers.
- LPS induced an up-regulation of $Ig\alpha_v$, $Ig\beta_3$, $Ig\beta_1$ and CD44 receptors in the rat SN and their expression on glial cells and macrophages.

In conclusion, neither endogenous nor exogenous OPN conferred any protection to dopaminergic cell lines against toxic insult although they expressed $Ig\alpha_v$, $Ig\beta_3$, $Ig\beta_1$ and CD44 receptors. However, OPN protected dopaminergic neurons both in primary VM culture and in rat SN against toxin induced cell death and showed a role in regulating the inflammatory process. Although the exact role of $Ig\alpha_v$, $Ig\beta_3$, $Ig\beta_1$ and CD44 receptors and glial cells is still not clear from these studies, the results provided a platform for further investigation of OPN's potential as a neuroprotective agent for PD.

The concept of employing endogenous molecules as neuroprotective agents is not novel. Various endogenous substances including proteins, hormones and cytokines have also been studied in relation to neuroprotection in PD. For example, estrogens showed neuroprotective properties in cell lines against oxidative stress (Singer *et al.*, 1998) and MPP^+ toxicity (Gélinas *et al.*, 2002). They were also protective in 6-OHDA and MPTP treated rodents (Kenchappa *et al.*, 2004; Quesada *et al.*, 2008) but no clinical trials have been undertaken to confirm their effectiveness. Endogenous molecules involved in inflammatory regulation have also showed some positive pre-clinical data but further investigations are still required. For example, IL-10 was shown to protect dopaminergic neurons of the VM culture from LPS toxicity (Qian *et al.*, 2006) and gene delivery of IL-10 into the rat striatum preserved dopaminergic neurons following 6-OHDA lesioning and reduced microglial activation (Johnston *et al.*, 2008). The endogenous peptide exendin-4 is a glucagon-like peptide 1 receptor agonist which has neurotrophic and antiapoptotic effects (Li *et al.*, 2003; Perry *et al.*, 2002). It showed neuroprotective effects in cultured cell lines and primary dopaminergic neurons (Li *et al.*, 2009) but also in rats when injected 7 days after LPS or 6-OHDA lesioning (Harkavyi *et al.*, 2008). Exendin-4 has the advantages of safety and BBB permeability (Harkavyi *et al.*, 2010) but whether it will show neuroprotection in clinical trials remains to be determined. As discussed in Chapter 1, neurotrophic factors have been explored as a potential neuroprotective treatment in PD. The neurotrophic factor GDNF showed promising results in pre-clinical studies but more clinical trials are required to determine whether it shows efficacy in PD. Neurturin, a neurotrophic factor member of the GDNF family, has also been tested for neuroprotection. Neurturin exhibited protective properties in VM cultures (Horger *et al.*, 1998) and protected nigral dopaminergic neurons from 6-OHDA induced cell death (Horger *et al.*, 1998; Rosenblad *et al.*, 1999), medial forebrain bundle axotomy in rats (Tseng *et al.*, 1998) and MPTP challenge in monkeys (Kordower *et al.*, 2006). Recently, a phase I clinical trial confirmed safety and efficacy of neurturin delivered via an AAV vector (CERE-120) into the putamen of PD patients (Marks Jr *et al.*, 2008) and was followed by a phase II double blind randomised clinical trial using the same delivery method. This trial did not meet its primary endpoint of efficacy but positive improvements were seen in a subgroup of patients who were followed for a longer period of time (Marks Jr *et al.*, 2010). Thus, neurotrophic factors have shown promise as neuroprotectants in PD but this has not

yet been translated into a proven clinical effect. For this reason, OPN would seem to offer an alternative approach through endogenous mechanisms as shown in this thesis. However, a lack of translation from the laboratory to the clinic has dogged the development of neuroprotective drugs and this raises questions about the experimental procedures that are used as discussed below. In addition, a number of questions arise from findings in the current studies and they are also discussed below.

6.3 Are cell culture and experimental models of dopaminergic cell death in PD suitable for testing neuroprotective agents?

In these studies, OPN was investigated for protective effects using cell lines, primary VM culture and LPS lesioned rats. Results obtained in cell lines did not agree with those in primary culture and rats, suggesting that this may be due to differences in models. In addition, a number of agents such as those discussed in Section 1.1.1 showed protective properties in cultured cells and animals but when tested in man did not show the same effects. Therefore, it is possible that these models do not closely mimic the complex pathology leading to neuronal degeneration in PD. This is discussed below in relation to results obtained in the current studies.

6.3.1 *In-vitro* models of nigral cell death

In the present studies, OPN showed protective effects in the primary VM culture and in rats but not in dopaminergic cell lines. Similar findings were previously reported, where neuroprotective agents that were effective *in-vivo*, did not have the same effect on SH-SY5Y cell lines (Kou *et al.*, 2008; Presgraves *et al.*, 2004). Conversely, other neuroprotective agents have showed neuroprotection in cell lines and subsequently in other models, for example dopaminergic agonists, MAO-B inhibitors, anti-oxidants and anti-apoptotic agents (Mandel *et al.*, 2003; Schapira, 2008b). Although factors related to OPN itself may be underlying this discrepancy as discussed in Chapter 3, it may also be due to the nature of cell lines.

Cell lines constitute an important tool for screening potential new drugs for PD. They offer the advantages of shorter experiment time and being less labour intensive than primary culture or animals. Dopaminergic cell lines are an unlimited

source of mono-typic cells with similar functional and biochemical characteristics to dopaminergic neurons. They can also be useful in studying mechanisms of action via pharmacological manipulations but also studying protein functions by overexpressing specific targets or silencing specific proteins. However, these cell lines are obviously not authentic dopaminergic neurons and this already presents a number of issues. For instance, SH-SY5Y cells are resultant of Ras mutation leading to chronic activation of MAPK/ERK signaling and therefore may exhibit different mechanisms of cell death to mature neurons (Abramova *et al.*, 2002). In addition, SH-SY5Y cells exhibit low dopamine synthesis enzymes and DAT (Presgraves *et al.*, 2004). Therefore these cell lines may respond differently to exogenous stimuli from mature neurons. In addition, cell lines are continuously dividing, meaning that the number of cells is increasing during the course of the experiment and this makes it difficult to distinguish whether the toxin or the protective agent are affecting cell death or proliferation rate (Datki *et al.*, 2003). By contrast, neurons in the brain are not able to divide with little ability of recovery from injury and are therefore highly vulnerable (Klöcker *et al.*, 2001). This questions how well can results from cell lines be representative of what might occur in brain neurons. In addition, some cells of the dopaminergic neuroblastoma cell lines can lose their neuronal or dopaminergic characteristics especially at higher passage numbers. This was overcome in these studies by using low passage numbers and regularly staining for the dopaminergic marker TH. Furthermore, the EC_{50} of MPP^+ was 100 fold greater in cell lines compared to primary dopaminergic neurons (Chapter 3) and this could be indicative of lower sensitivity of cell lines. Indeed, cell lines previously showed lower sensitivity to both toxins and protective agents than primary mesencephalic neurons (Storch *et al.*, 2000). This maybe a result of the immortalisation process, suggesting that cell lines may be less accurate in predicting physiological outcomes in intact brains.

Primary VM culture has some advantages over dopaminergic cell lines. It offers authentic dopaminergic neurons which have not been genetically modified to become immortalised. Also, these dopaminergic neurons are cultured within a more heterogenous, physiological environment with other cell types such as other neuronal and glial cells which are also present in the intact SN. However, this culture also carries the limitations of low percentage of dopaminergic neurons (3-5%) and

increased vulnerability of neurons the longer they are cultured *in-vitro*. The VM culture is also limited in terms of culture life span and passaging, rendering some useful manipulations, such as stable transfections, impossible to achieve. In addition, the limited culture life span imposes neuroprotection assays with a very fast time-course of cell death compared to the slow degeneration of neurons in PD.

In the current studies, there was an interesting difference between primary VM culture and rat SN; Igα_v, Igβ_3, Igβ_1 and CD44 were expressed on neurons and glia in VM culture but only on neurons in naive rat SN. This may be due to the nature of the VM culture model as the cultured cells are embryonic in nature and in the process of development. In fact, integrin receptors are expressed in developing cells due to their roles in survival, adhesion, migration and proliferation (Anthis *et al.*, 2011). This may also be the case for the CD44 receptor as it is also involved in these vital processes. Therefore, mature glial cells may stop expressing these receptors and only up-regulate them in the event of injury or inflammation when adhesion, migration and proliferation activities are required as was the case in this study. Consequently, although VM culture contains glial cells their phenotype may be different to mature glia in the SN.

In summary, cell line or tissue culture models provide a good starting point to investigate properties of protective agents, though it remains difficult to interpret how data from *in-vitro* toxin models will relate to the complex pathophysiology of PD.

6.3.2 *In-vivo* models of nigral cell death

Testing neuroprotective candidates in animals may be more physiologically relevant than *in-vitro* systems. However, none of the toxin-based animal models available mimic the exact pathophysiological features of PD and they all have advantages and shortcomings. Therefore the choice of animal model is usually based on the type of investigation required. Here, the LPS model was chosen since the objective of these studies was to look at inflammation mediated neurodegeneration for which LPS is the most suitable model. The 6-OHDA model, for instance, could have also been used as it induces an inflammatory reaction (Cicchetti *et al.*, 2002; He *et al.*, 2001) but the mechanism of cell death is through accumulation of the toxin in dopaminergic neurons and subsequent oxidisation into hydrogen peroxide and

paraquinone which mediate cellular damage (Saner *et al.*, 1971). The following discussion focuses on the use of LPS and its relation to PD pathology.

LPS is not directly toxic to neurons but induces degeneration via a reactive gliosis and inflammation (Gao *et al.*, 2002; Taylor *et al.*, 2003). However, in PD, it is not known whether inflammation is a primary event or is secondary to neuronal degeneration (Chapter 1). Although LPS can cause dopaminergic neuron loss from the SN and a reactive gliosis, its administration to the SN results in very acute cell death different to the progressive nature of neurodegeneration in PD. In this study, significant dopaminergic cell death was seen 1 day after LPS administration and previously, significant cell death was observed as early as 16hr post lesioning (Iravani *et al.*, 2002). In addition, the destruction of the nigro-striatal system remains static up to 1 year post lesioning (Herrera *et al.*, 2000). This disadvantage, however, is common to most toxin based animal models with varying extents. Systemic infusion of LPS may cause a more progressive cell death but it does cause widespread inflammation in the brain and may not be a model specific to PD (Qin *et al.*, 2007). Chronic administration of LPS into the SN over 2 weeks was shown in one study to produce progressive loss of dopaminergic neurons (Gao *et al.*, 2002). This, however, may be a transient progression resulting from prolonged administration of toxin. Besides, this model is not confirmed by other studies, thus not well established. Another limitation, is the fact that inflammation following LPS lesion is a transient process (Herrera *et al.*, 2000; Iravani *et al.*, 2005) whereas in PD there is on-going inflammation (McGeer *et al.*, 1988). This, however, may not affect the present study as the experiments were carried out within a one week period while inflammation is still present. Nevertheless, the pathological consequences of a transient inflammation might be different to that of an on-going one.

In order to better predict the outcome of administering OPN to humans with PD, the primate MPTP model may be more suitable. Non-human primates are more closely related to humans than rodents. As opposed to LPS nigral injection, inflammatory gliosis following MPTP treatment in primates is ongoing (Barcia *et al.*, 2004; Langston *et al.*, 1999). In addition, previous studies have shown that following LPS insult there is up-regulation of OPN, while in PD and MPTP-treated marmosets OPN expression is decreased (Iczkiewicz *et al.*, 2006). This suggests that regulation of OPN in MPTP-treated marmosets is similar to that in PD. However,

this may be because LPS was used in the early stage while MPTP treated monkeys were used after one year of MPTP challenge. Noteworthy, the cell death process induced by MPTP, like LPS, is acute and there is lack of inclusion bodies (Halliday *et al.*, 2009). Overall, there is no animal or cell culture model that exactly mimics the pathophysiology of PD. However, these *in-vitro* and *in-vivo* models represent valuable tools that can help study one or more biochemical aspects contributing to the complex process of cell death in PD, and provide a realistic, simple system to investigate protective agents before attempting clinical trials.

6.4 Are integrin and CD44 receptors important potential targets for the treatment of PD?

In the VM culture, OPN's protective effect was inhibited by non-specific integrin blockers suggesting that interaction of OPN with these receptors is essential to achieve neuroprotection. The CD44 receptor may also play a role, but this remains to be investigated. In addition, OPN was previously shown to have pro-survival effects in different cell and tissue types and this was mediated via integrins (Doyle *et al.*, 2008; Meller *et al.*, 2005), CD44 receptors (Caers *et al.*, 2006) or both (Lee *et al.*, 2007). Although the role of integrin and CD44 receptors in the protective actions of OPN in rats was not investigated here, results from VM culture in addition to previous reports suggest that one or more of these receptors are likely to be the mediators of OPN's actions. Therefore, a better understanding of the roles of these receptors may lead to discovering new potential neuroprotective strategies.

Integrin and CD44 receptors are ubiquitous transmembrane receptors involved in regulating vital functions such as adhesion, migration and proliferation (Chapter 1). They are up-regulated in inflammatory reactions in the CNS where they mediate inflammatory cell adhesion and proliferation as discussed in Section 5.4.2. In the current studies, presence of these receptors in the SN suggests that they may play an important role in PD. The up-regulation of these receptors following LPS insult to the SN supports this suggestion and may open the door for a new area of research in PD. Expression of integrin receptors on some glial cells in LPS-lesioned SN suggests that OPN may act on glial cells to mediate its protective actions possibly by reducing release of pro-inflammatory cytokines. These receptors are differentially activated by different ligands, some inducing pro-inflammatory and others anti-

inflammatory actions (Milner *et al.*, 2002) and targeting them via inhibitors seems to have anti-inflammatory effects in a number of disease models including arthritis (Homandberg *et al.*, 1994) and encephalomyelitis (Brocke *et al.*, 1999). This further emphasises the potential of targeting these receptors as a means of treating PD.

In addition, integrin receptors may be involved in neurotrophic factor signalling. Integrin β_1 is suggested to act as an alternative signalling receptor for GDNF (Cao *et al.*, 2008). In fact, GDNF enhances expression of integrin α_v (Funahashi *et al.*, 2003) and both GDNF and BDNF increase the expression of this integrin in primary dopaminergic neurons (Gao *et al.*, 2002). This suggests that neurotrophic factors may mediate their protective effects in dopaminergic neurons through integrin receptors. In support of this concept, GDNF mediated survival of dopaminergic neurons was inhibited by integrin-blocking peptides (Chao *et al.*, 2003). This area has not been extensively studied and future studies may reveal more important roles for integrin receptors in mediating neurotrophic factor effects. It is noteworthy here that OPN may also exert its protective effects through inducing neurotrophic factors particularly since unpublished studies in our laboratories showed that OPN induces the release of GDNF and BDNF in VM culture (Broom, 2011, personal communication).

Moreover, integrin receptors may have a role in neurogenesis. Increased integrin expression following neuronal injury is correlated with successful regeneration of peripheral neurons (Previtali *et al.*, 2001) and with limited regeneration of central neurons after neurotrophin treatment (Plantman *et al.*, 2005). A number of integrin subunits, including Igα_4, Igα_5, Igα_6, Igα_7 and Igβ_1, are upregulated in sensory and motor neurons during peripheral nerve regeneration (Lemons *et al.*, 2008). Further, dorsal root ganglia neurons that are subjected to a preconditioning injury, upregulate expression of Igα_5 and Igα_7 and subsequently show enhanced outgrowth when explanted in culture, suggesting a role of these integrins in neurogenesis (Ekström *et al.*, 2003; Gardiner *et al.*, 2007). Although, the current study was not looking at neurogenesis, the role of integrin receptors in this process is very interesting and may be worth future studies. The concept of pharmacologically inducing neurogenesis is receiving considerable interest but evidence for neurogenesis of dopaminergic neurons in basal ganglia is controversial and awaits clarification (Deierborg *et al.*, 2008).

Nevertheless, integrins α_v, β_3 and β_1 were expressed in all dopaminergic cell lines tested in this study, in neurons and glia of the VM culture and in neurons in the SN. In fact, integrin receptors are ubiquitously expressed not only in the CNS but also in the periphery. Targeting these receptors may thus be associated with unwanted, non-specific widespread effects. Therefore, there may be a need for finding subunits or subunit dimers that aggregate specifically in the SN. This may be difficult to achieve since it will require investigation of a large number of bodily tissues. Ligands of these receptors including OPN may also be ubiquitous multifunctional proteins, making the situation more complicated. Nonetheless, existing medicines such as aspirin are ubiquitous but also well tolerated. This issue requires careful investigations.

A limited number of these receptors were investigated in the current study ($Ig\alpha_v$, $Ig\beta_3$, $Ig\beta_1$) due to time and resource constraints, but there remain many other integrin receptors which may have different expression patterns to the currently investigated ones and may also have important roles in PD. For instance, integrins involved in neurogenesis include $Ig\alpha_4$, $Ig\alpha_5$, $Ig\alpha_6$, $Ig\alpha_7$ as discussed above. Although receptors investigated here were shown to mediate pro-survival effects, OPN can bind to at least 7 different integrin receptors (Chapter 1) and not all of these have been tested. Further investigations into the exact role of these receptors in PD may not only help in better understanding of the pathophysiology of the disease, but may also open possibilities for new neuroprotective strategies.

6.5 Is OPN a future treatment for PD?

One question arising from these studies is whether OPN would act differently in the human brain, particularly since effects of OPN varied in different models. In cell lines, no effects of this protein were observed and while it exhibited protective effects in both VM culture and rats, the underlying effects on inflammatory cells were different. OPN had a proliferating effect on microglia in VM culture, and a decrease in activated microglia in LPS treated rats. It is interesting, however, that in both models OPN only affected microglia with no significant effects on astrocytes or macrophages and this is consistent. In fact, in PD OPN was expressed in neurons and microglia (Iczkiewicz *et al.*, 2006). It is not clear why OPN did not have an effect on macrophages and astrocytes but it may be decreasing the release of NO

from these cells since it was previously shown to decrease NO release from macrophages (Crawford *et al.*, 1998) and this needs further investigation. The attenuated microgliosis in the rat LPS model is most probably protective, since activated microglia are discussed to have toxic effects on neurons (Chapter1) and LPS toxicity to neurons is a result of microglial activation and subsequent release of pro-inflammatory cytokines (Chapter 1). However, the proliferative effect on microglia in untreated VM cultures is not very well understood. This latter result should be treated with caution, as it is in line with the previously reported ability of OPN to recruit inflammatory cells to injury site (Giachelli *et al.*, 1998; Kawashima *et al.*, 1999; Murry *et al.*, 1994b; O'Regan *et al.*, 1999; Patarca *et al.*, 1989b). The difference in OPN effects may reflect the dissimilarity of models, as embryonic stage microglia cultured in an 'aritificial' environment may act differently to mature microglia in the intact SN. It is noteworthy, however, that OPN only induced proliferation of microglia with no activation suggesting a possible protective effect. Treatment with OPN may have signalled a protective response of the VM culture by increasing the number of microglia in order to release more neurotrophic factors. Indeed OPN was shown to increase neurotrophic factor release in VM cultures (Broom, 2011, personal communication). In support of this, growth factors have been shown to protect primary dopaminergic neurons against MPP^+ toxicity through glial cell stimulation and proliferation (Dalia *et al.*, 1993; Otto *et al.*, 1993; Park *et al.*, 1992).

Consequently, further steps should be taken to investigate the underlying inflammatory regulation by OPN such as effects on inflammatory cytokines and this may explain better the results of the current studies. This is particularly important since one recent study found that OPN deficient mice showed reduced striatal dopaminergic fibre loss after MPTP challenge (Maetzler *et al.*, 2007). In the same study, OPN levels were increased in serum and cerebrospinal fluid of PD patients compared to controls and higher OPN levels were associated with more severe motor symptoms (Maetzler *et al.*, 2007). Lower striatal dopaminergic fibre loss in MPTP-treated OPN null mice suggests that lack of OPN is protective, but this has not been confirmed by other studies. In contrast, the current study showed that OPN expressing or OPN lacking cell lines do not show differences in sensitivity to toxin. The increased OPN levels in body fluids from PD patients (Maetzler *et al.*, 2007)

does not necessarily suggest a harmful effect of the protein but may also indicate that it is a protective homeostatic reaction. The pathophysiology in PD is considerably different to the models employed in the current study and much more complex as discussed in Chapter 1. Therefore, although the overall expected effect in the human brain based on these studies would be a protective one, further studies are required before this conclusion can be made.

Another important issue to consider is the implication of OPN in cancer. OPN is up-regulated in a number of cancers and higher levels are associated with a metastatic phenotype (Chapter1). Indeed, there is a close relationship between molecules that induce cell survival and tumorigenesis. The involvement of OPN in inflammation and cell survival may be related to tumour pathology since it is consistent with the notion that tumours are 'wounds that do not heal'(Balkwill *et al.*, 2001) and 'tissues that never cease to develop' (Pollard, 2004). In fact, some suggest that PD patients may be protected from some cancers but have higher incidence of other ones including melanoma and breast cancer (Inzelberg *et al.*, 2007), suggesting predisposition of parkinsonian patients to developing these cancers or a pathological link between PD and tumours. Therefore, although in this study treatment of cultured or nigral dopaminergic cells with OPN did not induce neuronal proliferation, the consequences of administering OPN into SN in PD should be considered very carefully.

Noteworthy, in the studies described here, OPN treatment was always introduced before toxic insult and this was because the toxins used induced very acute cell death. Had OPN treatment been given after toxic insult, the cells may have already died or been severely damaged and this would be testing whether OPN has neurorestorative properties. However, the aim of this study was to test whether OPN would *protect* the cells from dying. This is because PD is progressive in nature and dopaminergic neurone loss is slow and on-going and the aim of neuroprotective agents is to be administered in early-diagnosed patients to prevent further loss of dopaminergic neurons from the SN and deterioration of symptoms. Of course, there remains the possibility of future investigations of neuro-restorative properties. PD is not diagnosed until considerable dopaminergic neurodegeneration had taken place (Agid, 1991; Fearnley *et al.*, 1991), and a relevant question is whether an anti-inflammatory effect at this stage would protect the remaining dopaminergic neurons

from undergoing degeneration. Importantly, the use of NSAIDS is associated with lower risk of PD according to epidemiological studies (Chapter 1), suggesting that early prevention of an inflammatory reaction in the SN may protect against degeneration of dopaminergic neurons. In addition, pre-clinical studies in animals showed protective effects of anti-inflammatory agents in toxin induced nigral cell death (Chapter 1). However, there is no clinical evidence that anti-inflammatory agents given after the diagnosis of PD are neuroprotective. Perhaps an anti-inflammatory effect of OPN coupled with other pro-survival effects such as its ability to inhibit apoptosis would be more effective in preventing further loss of dopaminergic neurons if administered at early stages of the disease. Overall, OPN showed promising results as a potential neuroprotective agent but further studies are required to clarify the underlying mechanism and to test safety.

6.6 Further development of OPN as a treatment for PD

The above discussion highlights few areas that require further investigations in order to establish the role of OPN as a potential protective agent. First, testing whether OPN would be protective in MPTP treated primates as this may mimic more closely the human disease. Second, further investigating the mechanisms that may be involved in the protective effects of OPN such as anti-apoptotic, anti-oxidant and anti-inflammatory effects. Third, studying the role of CD44 and specific integrin receptors in the protective effects of OPN and elucidating the receptors and functional fragments of the protein important for this effect. Further, other parts of the protein, not studied here, may have intrinsic protective actions and are worth future investigations. For instance, OPN has a calcium binding site and is reported to bind this cation (Chapter 1) suggesting that it may also be able to bind other divalent metals implicated in PD pathology such as iron. Calcium itself is suggested to play a role in neuronal death after accumulation in the neuron (Landfield *et al.*, 1992). Its role in dopaminergic cell death is supported by evidence of protective effects of the calcium buffering protein calbindin in the dopaminergic cell line PC12 (McMahon *et al.*, 1998). Further, the use of dihydropyridine calcium channel blockers as anti-hypertensives was associated with lower risk of PD (Rodnitzky, 1999). Recently, new cell binding sequences have been identified in OPN which may play a role in cell survival but receptors for these segments are still unknown (Rittling, 2011).

In addition to the above, administration of OPN also requires further development. One of the major obstacles which could negatively impact the potential of using OPN as an effective neuroprotective and clinically viable candidate is its proteinaceous nature which renders it susceptible to first pass metabolism in addition to limiting its transport across the blood brain barrier. OPN would require invasive surgery to be delivered to the SN which can be achieved via direct administration of the protein or delivery of the gene by means of a viral vector. However, this would be a major limitation to the use of OPN as a treatment in PD. Several strategies have been proposed to overcome these shortcomings. Aerosolising the protein for nasal drug delivery could be a better alternative considering the advantageous absorption rates and improved bioavailability. However, OPN might also exhibit limited blood-brain barrier penetrability considering its molecular size and charged polar surface.

The use of permeation enhancers such as protease inhibitors as additives to attain consistently high bioavailability levels has gained increasing interest in recent reports (Morishita *et al.*, 2006). However, the applicability is questioned due to the potential toxic effects of protease blockers and the implications of polypharmacy. Another important aspect which can be exploited, is formulating OPN using advanced drug delivery systems into a dosage form which is administered orally or parenterally in order to improve stability and targetability. These systems include biodegradable polymeric conjugates and nano- and microparticulate carriers (microreservoir systems such as liposomes and micellar structures) which offer the advantage of controlling the rate of release in addition to localising the delivery of active pharmaceutical which can be site-specific (Gombotz *et al.*, 1995).

Importantly, A 15-mer fragment of OPN has also been shown here to be protective in MPP^+ treated VM culture and in a previous study to be protective in rats against LPS and 6-OHDA (Iczkiewicz *et al.*, 2010). This fragment has advantages for systemic administration over the full length protein. It is less likely to be metabolised and its smaller size means improved penetrability to the BBB. The intranasal administration of a 13-mer fragment in mice has already been reported to provide neuroprotection in a stroke model (Doyle *et al.*, 2008). However, the effect of the 15-mer fragment was partial in this study and there is a possibility that other parts of the protein are protective or essential for full function of the protein.

Therefore, further investigations are required to identify segments of the protein which can offer the full spectrum of protective effects.

One distinct approach which merits further investigation is designing peptidomimetics which offer comparable pharmacological potency but better bioavailability. The design of pseudopeptide/non-peptide analogues requires the modification of physicochemical characteristics of the protein or fragment in order to overcome the physical and biochemical barrier properties of endogenous membranes (Pauletti *et al.*, 1997). The design of an effective peptidomimetic or a non-peptide analogue requires the application of various design strategies to understand the topographical and stereoelectronic properties of the pharmacophore and side chain moieties (Pauletti *et al.*, 1997).

6.7 Conclusion

As hypothesized, OPN seems to be a promising potential neuroprotective agent, worth further investigation. The potential of small peptide fragments carrying the same effect as the parent protein and the possibility of synthesizing peptidomimetics gives OPN an advantage over the currently investigated endogenous proteins. The ability of OPN to protect neurons from inflammation mediated neurodegeneration makes it also a potential treatment candidate for other neurodegenerative diseases with evident inflammatory involvement such as Alzheimer's disease.

References

Abramova NA, Cassarino DS, Khan SM, Painter TW, Bennett JP, Jr. (2002). Inhibition by R(+) or S(-) pramipexole of caspase activation and cell death induced by methylpyridinium ion or beta amyloid peptide in SH-SY5Y neuroblastoma. *Journal of neuroscience research* **67**(4)**:** 494-500.

Agid Y (1991). Parkinson's disease: pathophysiology. *Lancet* **337**(8753)**:** 1321-1324.

Agnihotri R, Crawford HC, Haro H, Matrisian LM, Havrda MC, Liaw L (2001). Osteopontin, a novel substrate for matrix metalloproteinase-3 (stromelysin-1) and matrix metalloproteinase-7 (matrilysin). *J Biol Chem* **276**(30)**:** 28261-28267.

Ahlskog JE (2005). Challenging conventional wisdom: the etiologic role of dopamine oxidative stress in Parkinson's disease. *Mov Disord* **20**(3)**:** 271-282.

Ahlskog JE, Muenter MD (2001). Frequency of levodopa-related dyskinesias and motor fluctuations as estimated from the cumulative literature. *Mov Disord* **16**(3)**:** 448-458.

Akiyama H, Kawamata T, Dedhar S, McGeer PL (1991). Immunohistochemical localization of vitronectin, its receptor and beta-3 integrin in Alzheimer brain tissue. *Journal of Neuroimmunology* **32**(1)**:** 19-28.

Akiyama H, McGeer PL (1990). Brain microglia constitutively express [beta]-2 integrins. *Journal of Neuroimmunology* **30**(1)**:** 81-93.

Akiyama H, Tooyama I, Kawamata T, Ikeda K, McGeer PL (1993). Morphological diversities of CD44 positive astrocytes in the cerebral cortex of normal subjects and patients with Alzheimer's disease. *Brain Research* **632**(1-2)**:** 249-259.

Alam ZI, Daniel SE, Lees AJ, Marsden DC, Jenner P, Halliwell B (1997). A generalised increase in protein carbonyls in the brain in Parkinson's but not incidental Lewy body disease. *J Neurochem* **69**(3)**:** 1326-1329.

Allan SM, Rothwell NJ (2001). Cytokines and acute neurodegeneration. *Nat Rev Neurosci* **2**(10)**:** 734-744.

Aloisi F (1999). The role of microglia and astrocytes in CNS immune surveillance and immunopathology. *Adv Exp Med Biol* **468:** 123-133.

Aloisi F, De Simone R, Columba-Cabezas S, Levi G (1999). Opposite effects of interferon-gamma and prostaglandin E2 on tumor necrosis factor and interleukin-10 production in microglia: a regulatory loop controlling microglia pro- and anti-inflammatory activities. *Journal of neuroscience research* **56**(6)**:** 571-580.

Alves G, Forsaa EB, Pedersen KF, Dreetz Gjerstad M, Larsen JP (2008). Epidemiology of Parkinson's disease. *J Neurol* **255 Suppl 5:** 18-32.

Amano T, Richelson E, Nirenberg M (1972). Neurotransmitter Synthesis by Neuroblastoma Clones Vol. 69, pp 258-263.

Amendola R, Martinez R, Negroni A, Venturelli D, Tanno B, Calabretta B, *et al.* (2001). DR-nm23 expression affects neuroblastoma cell differentiation, integrin expression, and adhesion characteristics Vol. 36, pp 93-96.

Amy M, Cynthia MvG, Bhumsoo K, Kenneth LvG, Eva LF (2004). Integrin Expression Regulates Neuroblastoma Attachment and Migration. *Neoplasia* **6:** 332-342.

Anglade P, Vyas S, Javoy-Agid F, Herrero MT, Michel PP, Marquez J, *et al.* (1997). Apoptosis and autophagy in nigral neurons of patients with Parkinson's disease. *Histol Histopathol* **12**(1)**:** 25-31.

Angulo A, Suto C, Boehm MF, Heyman RA, Ghazal P (1995). Retinoid activation of retinoic acid receptors but not of retinoid X receptors promotes cellular differentiation and replication of human cytomegalovirus in embryonal cells Vol. 69, pp 3831-3837.

Anthis NJ, Campbell ID (2011). The tail of integrin activation. *Trends in Biochemical Sciences* **36**(4)**:** 191-198.

Ara J, Przedborski S, Naini AB, Jackson-Lewis V, Trifiletti RR, Horwitz J, *et al.* (1998). Inactivation of tyrosine hydroxylase by nitration following exposure to peroxynitrite and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *Proc Natl Acad Sci U S A* **95**(13)**:** 7659-7663.

Arimoto T, Bing G (2003). Up-regulation of inducible nitric oxide synthase in the substantia nigra by lipopolysaccharide causes microglial activation and neurodegeneration. *Neurobiol Dis* **12**(1)**:** 35-45.

Aruffo A, Stamenkovic I, Melnick M, Underhill CB, Seed B (1990). CD44 is the principal cell surface receptor for hyaluronate. *Cell* **61**(7)**:** 1303-1313.

Ashizawa N, Graf K, Do YS, Nunohiro T, Giachelli CM, Meehan WP, *et al.* (1996). Osteopontin Is Produced by Rat Cardiac Fibroblasts and Mediates AII-induced DNA Synthesis and Collagen Gel Contraction Vol. 98, pp 2218-2227.

Ashkar S, Glimcher MJ, Saavedra RA (1993a). Mouse osteopontin expressed in E. coli exhibits autophosphorylating activity of tyrosine residues. *Biochem Biophys Res Commun* **194**(1)**:** 274-279.

Ashkar S, Teplow DB, Glimcher MJ, Saavedra RA (1993b). In vitro phosphorylation of mouse osteopontin expressed in E. coli. *Biochem Biophys Res Commun* **191**(1)**:** 126-133.

Ashkar S, Weber GF, Panoutsakopoulou V, Sanchirico ME, Jansson M, Zawaideh S, *et al.* (2000). Eta-1 (Osteopontin): An Early Component of Type-1 (Cell-Mediated) Immunity Vol. 287, pp 860-864.

Asplin JR, Arsenault D, Parks JH, Coe FL, Hoyer JR (1998). Contribution of human uropontin to inhibition of calcium oxalate crystallization. *Kidney Int* **53**(1)**:** 194-199.

Attur MG, Dave MN, Clancy RM, Patel IR, Abramson SB, Amin AR (2000). Functional Genomic Analysis in Arthritis-Affected Cartilage: Yin-Yang Regulation of Inflammatory Mediators by {alpha}5{beta}1 and {alpha}V{beta}3 Integrins Vol. 164, pp 2684-2691.

Attur MG, Dave MN, Stuchin S, Kowalski AJ, Steiner G, Abramson SB, *et al.* (2001). Osteopontin: an intrinsic inhibitor of inflammation in cartilage. *Arthritis Rheum* **44**(3)**:** 578-584.

Balkwill F, Mantovani A (2001). Inflammation and cancer: back to Virchow? *The Lancet* **357**(9255)**:** 539-545.

Barcia C, Bahillo AS, Fernández-Villalba E, Bautista V, Poza Y Poza M, Fernández-Barreiro A, *et al.* (2004). Evidence of active microglia in substantia nigra pars compacta of parkinsonian monkeys 1 year after MPTP exposure. *Glia* **46**(4)**:** 402-409.

Barczyk M, Carracedo S, Gullberg D (2010). Integrins. *Cell and Tissue Research* **339**(1)**:** 269-280.

Barron KD (1995). The microglial cell. A historical review. *J Neurol Sci* **134 Suppl:** 57-68.

Barry ST, Ludbrook SB, Murrison E, Horgan CMT (2000). Analysis of the [alpha]4[beta]1 Integrin-Osteopontin Interaction. *Experimental Cell Research* **258**(2)**:** 342-351.

Batchelor PE, Liberatore GT, Wong JY, Porritt MJ, Frerichs F, Donnan GA, *et al.* (1999). Activated macrophages and microglia induce dopaminergic sprouting in the injured striatum and express brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor. *J Neurosci* **19**(5)**:** 1708-1716.

Bayless KJ, Davis GE (2001). Identification of Dual alpha 4beta 1 Integrin Binding Sites within a 38 Amino Acid Domain in the N-terminal Thrombin Fragment of Human Osteopontin Vol. 276, pp 13483-13489.

Bayless KJ, Meininger GA, Scholtz JM, Davis GE (1998). Osteopontin is a ligand for the alpha4beta1 integrin Vol. 111, pp 1165-1174.

Beal MF, Matthews RT, Tieleman A, Shults CW (1998). Coenzyme Q10 attenuates the 1-methyl-4-phenyl-1,2,3,tetrahydropyridine (MPTP) induced loss of striatal dopamine and dopaminergic axons in aged mice. *Brain Res* **783**(1)**:** 109-114.

Beckman JS, Koppenol WH (1996). Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *American Journal of Physiology - Cell Physiology* **271**(5)**:** C1424-C1437.

Bekris LM, Mata IF, Zabetian CP (2010). The genetics of Parkinson disease. *Journal of geriatric psychiatry and neurology* **23**(4)**:** 228-242.

Bellahcene A, Castronovo V, Ogbureke KUE, Fisher LW, Fedarko NS (2008). Small integrin-binding ligand N-linked glycoproteins (SIBLINGs): multifunctional proteins in cancer. *Nat Rev Cancer* **8**(3)**:** 212-226.

Bender A, Krishnan KJ, Morris CM, Taylor GA, Reeve AK, Perry RH, *et al.* (2006). High levels of mitochondrial DNA deletions in substantia nigra neurons in aging and Parkinson disease. *Nat Genet* **38**(5)**:** 515-517.

Bezard E, Gross CE, Brotchie JM (2003). Presymptomatic compensation in Parkinson's disease is not dopamine-mediated. *Trends Neurosci* **26**(4)**:** 215-221.

Biedler JL, Helson L, Spengler BA (1973). Morphology and Growth, Tumorigenicity, and Cytogenetics of Human Neuroblastoma Cells in Continuous Culture Vol. 33, pp 2643-2652.

Biedler JL, Roffler-Tarlov S, Schachner M, Freedman LS (1978). Multiple Neurotransmitter Synthesis by Human Neuroblastoma Cell Lines and Clones Vol. 38, pp 3751-3757.

Blum D, Torch S, Lambeng N, Nissou M, Benabid AL, Sadoul R, *et al.* (2001). Molecular pathways involved in the neurotoxicity of 6-OHDA, dopamine and MPTP: contribution to the apoptotic theory in Parkinson's disease. *Prog Neurobiol* **65**(2)**:** 135-172.

Blum D, Wu Y, Nissou MF, Arnaud S, Benabid AL, Verna JM (1997). p53 and Bax activation in 6-hydroxydopamine-induced apoptosis in PC12 cells. *Brain Research* **751**(1)**:** 139-142.

Bo L, Peterson JW, Mork S, Hoffman PA, Gallatin WM, Ransohoff RM, *et al.* (1996). Distribution of immunoglobulin superfamily members ICAM-1, -2, -3, and the beta 2 integrin LFA-1 in multiple sclerosis lesions. *J Neuropathol Exp Neurol* **55**(10)**:** 1060-1072.

Boka G, Anglade P, Wallach D, Javoy-Agid F, Agid Y, Hirsch EC (1994). Immunocytochemical analysis of tumor necrosis factor and its receptors in Parkinson's disease. *Neuroscience Letters* **172**(1-2)**:** 151-154.

Bolanos JP, Heales SJ, Peuchen S, Barker JE, Land JM, Clark JB (1996). Nitric oxide-mediated mitochondrial damage: a potential neuroprotective role for glutathione. *Free Radic Biol Med* **21**(7)**:** 995-1001.

Bonifati V, Rizzu P, van Baren MJ, Schaap O, Breedveld GJ, Krieger E, *et al.* (2003). Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. *Science* **299**(5604)**:** 256-259.

Bonvini JM, Schatzmann U, Beck-Schimmer B, Sun LK, Rittling SR, Denhardt DT, *et al.* (2000). Lack of in vivo function of osteopontin in experimental anti-GBM nephritis. *J Am Soc Nephrol* **11**(9)**:** 1647-1655.

Borges K, Gearing M, Rittling S, Sorensen ES, Kotloski R, Denhardt DT, *et al.* (2008). Characterization of osteopontin expression and function after status epilepticus. *Epilepsia* **49**(10)**:** 1675-1685.

Braak H, Ghebremedhin E, Rüb U, Bratzke H, Del Tredici K (2004). Stages in the development of Parkinson's disease-related pathology. *Cell and Tissue Research* **318**(1)**:** 121-134.

Braak H, Tredici KD, Rub U, de Vos RAI, Jansen Steur ENH, Braak E (2003). Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiology of Aging* **24**(2)**:** 197-211.

Brannan T, Yahr MD (1995). Comparative study of selegiline plus L-dopa-carbidopa versus L-dopa-carbidopa alone in the treatment of Parkinson's disease. *Ann Neurol* **37**(1)**:** 95-98.

Bredesen DE, Rao RV, Mehlen P (2006). Cell death in the nervous system. *Nature* **443**(7113)**:** 796-802.

Breidert T, Callebert J, Heneka MT, Landreth G, Launay JM, Hirsch EC (2002). Protective action of the peroxisome proliferator-activated receptor-γ agonist pioglitazone in a mouse model of Parkinson's disease. *Journal of Neurochemistry* **82**(3)**:** 615-624.

Brocke S, Piercy C, Steinman L, Weissman IL, Veromaa T (1999). Antibodies to CD44 and integrin alpha4, but not L-selectin, prevent central nervous system inflammation and experimental encephalomyelitis by blocking secondary leukocyte recruitment. *Proc Natl Acad Sci U S A* **96**(12)**:** 6896-6901.

Brodsky JL, McCracken AA (1997). ER-associated and proteasomemediated protein degradation: how two topologically restricted events came together. *Trends in Cell Biology* **7**(4)**:** 151-156.

Bukhatwa S, Iravani MM, Zeng B-Y, Cooper JD, Rose S, Jenner P (2009). An immunohistochemical and stereological analysis of PSI-induced nigral neuronal degeneration in the rat. *Journal of Neurochemistry* **109**(1)**:** 52-59.

Bukhatwa S, Zeng BY, Rose S, Jenner P (2010a). A comparison of changes in proteasomal subunit expression in the substantia nigra in Parkinson's disease, multiple system atrophy and progressive supranuclear palsy. *Brain Res* **1326:** 174-183.

Bukhatwa S, Zeng BY, Rose S, Jenner P (2010b). The effects of dose and route of administration of PSI on behavioural and biochemical indices of neuronal degeneration in the rat brain. *Brain Res* **1354:** 236-242.

Burdo TH, Wood MR, Fox HS (2007). Osteopontin prevents monocyte recirculation and apoptosis Vol. 81, pp 1504-1511.

Burns RS, Chiueh CC, Markey SP, Ebert MH, Jacobowitz DM, Kopin IJ (1983). A primate model of parkinsonism: selective destruction of dopaminergic neurons in the pars compacta of the substantia nigra by N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Proceedings of the National Academy of Sciences* **80**(14)**:** 4546-4550.

Butler WT (1995). Structural and functional domains of osteopontin. *Annals of the New York Academy of Sciences* **760:** 6-11.

Caers J, Gunthert U, Raeve H, Valckenborgh EV, Menu E, Riet IV, *et al.* (2006). The involvement of osteopontin and its receptors in multiple myeloma cell survival, migration and invasion in the murine 5T33MM model Vol. 132, pp 469-477.

Calabresi P, Picconi B, Parnetti L, Di Filippo M (2006). A convergent model for cognitive dysfunctions in Parkinson's disease: the critical dopamine-acetylcholine synaptic balance. *The Lancet Neurology* **5**(11)**:** 974-983.

Caltabiano S, Hum W-T, Attwell GJ, Gralnick DN, Budman LJ, Cannistraci A, *et al.* (1999). The integrin specificity of human recombinant osteopontin. *Biochemical Pharmacology* **58**(10)**:** 1567-1578.

Canu N, Barbato C, Ciotti MT, Serafino A, Dus L, Calissano P (2000). Proteasome Involvement and Accumulation of Ubiquitinated Proteins in Cerebellar Granule Neurons Undergoing Apoptosis Vol. 20, pp 589-599.

Cao J-P, Yu J-K, Li C, Sun Y, Yuan H-H, Wang H-J, *et al.* (2008). Integrin β1 is involved in the signaling of glial cell line-derived neurotrophic factor. *The Journal of Comparative Neurology* **509**(2)**:** 203-210.

Cardoso SM, Moreira PI, Agostinho P, Pereira C, Oliveira CR (2005). Neurodegenerative pathways in Parkinson's disease: therapeutic strategies. *Curr Drug Targets CNS Neurol Disord* **4**(4)**:** 405-419.

Castano A, Herrera AJ, Cano J, Machado A (2002). The degenerative effect of a single intranigral injection of LPS on the dopaminergic system is prevented by dexamethasone, and not mimicked by rh-TNF-α, IL-1β and IFN-&#x03B3. *Journal of Neurochemistry* **81**(1)**:** 150-157.

Castano A, Herrera AJ, Cano J, Machado A (1998). Lipopolysaccharide intranigral injection induces inflammatory reaction and damage in nigrostriatal dopaminergic system. *J Neurochem* **70**(4)**:** 1584-1592.

Chabas D, Baranzini SE, Mitchell D, Bernard CC, Rittling SR, Denhardt DT, *et al.* (2001). The influence of the proinflammatory cytokine, osteopontin, on autoimmune demyelinating disease. *Science* **294**(5547)**:** 1731-1735.

Chamak B, Mallat M (1991). Fibronectin and laminin regulate thein vitro differentiation of microglial cells. *Neuroscience* **45**(3)**:** 513-527.

Chang PL, Prince CW (1991). 1 alpha,25-dihydroxyvitamin D3 stimulates synthesis and secretion of nonphosphorylated osteopontin (secreted phosphoprotein 1) in mouse JB6 epidermal cells. *Cancer Res* **51**(8)**:** 2144-2150.

Chao CC, Ma YL, Chu KY, Lee EH (2003). Integrin alphav and NCAM mediate the effects of GDNF on DA neuron survival, outgrowth, DA turnover and motor activity in rats. *Neurobiol Aging* **24**(1)**:** 105-116.

Chen H, Zhang SM, Hernan MA, Schwarzschild MA, Willett WC, Colditz GA, *et al.* (2003a). Nonsteroidal anti-inflammatory drugs and the risk of Parkinson disease. *Arch Neurol* **60**(8)**:** 1059-1064.

Chen PS, Peng GS, Li G, Yang S, Wu X, Wang CC, *et al.* (2006). Valproate protects dopaminergic neurons in midbrain neuron//glia cultures by stimulating the release of neurotrophic factors from astrocytes. *Mol Psychiatry* **11**(12)**:** 1116-1125.

Chen W, Ma Q, Suzuki H, Hartman R, Tang J, Zhang JH (2011). Osteopontin reduced hypoxia-ischemia neonatal brain injury by suppression of apoptosis in a rat pup model. *Stroke* **42**(3)**:** 764-769.

Chen Y, Bal BS, Gorski JP (1992). Calcium and collagen binding properties of osteopontin, bone sialoprotein, and bone acidic glycoprotein-75 from bone. *Journal of Biological Chemistry* **267**(34)**:** 24871-24878.

Chen Y, Rittling S (2003b). Novel murine mammary epithelial cell lines that form osteolytic bone metastases: Effect of strain background on tumor homing. *Clinical and Experimental Metastasis* **20**(2)**:** 111-120.

Cherubini A, Hofmann G, Pillozzi S, Guasti L, Crociani O, Cilia E, *et al.* (2005). Human ether-a-go-go-related Gene 1 Channels Are Physically Linked to {beta}1 Integrins and Modulate Adhesion-dependent Signaling Vol. 16, pp 2972-2983.

Cheung NS, Hickling YM, Beart PM (1997). Development and survival of rat embryonic mesencephalic dopaminergic neurones in serum-free, antioxidant-rich primary cultures. *Neurosci Lett* **233**(1)**:** 13-16.

Choi ES, Rettig WJ, Wayner EA, Srour ML, Clegg DO (1994). Functional identification of integrin laminin receptors that mediate process outgrowth by human SY5Y neuroblastoma cells. *Journal of neuroscience research* **37**(4)**:** 475-488.

Choi JS, Kim HY, Cha JH, Choi JY, Lee MY (2007). Transient microglial and prolonged astroglial upregulation of osteopontin following transient forebrain ischemia in rats. *Brain Res* **1151:** 195-202.

Choi JS, Park HJ, Cha JH, Chung JW, Chun MH, Lee MY (2003). Induction and temporal changes of osteopontin mRNA and protein in the brain following systemic lipopolysaccharide injection. *J Neuroimmunol* **141**(1-2)**:** 65-73.

Christensen B, Nielsen MS, Haselmann KF, Petersen TE, Sorensen ES (2005). Post-translationally modified residues of native human osteopontin are located in clusters:

identification of 36 phosphorylation and five O-glycosylation sites and their biological implications. *The Biochemical journal* **390**(Pt 1)**:** 285-292.

Chuluyan HE, Lang BJ, Issekutz AC (2000). Differential mechanisms of neutrophil and monocyte adhesion on neuroblastoma cells: CD18 and VLA-4 integrins mediate adhesion to SK-N-SH, but not to SK-n-MC cell line Vol. 60, pp 649-655.

Chuluyan HE, Lang BJ, Yoshimura T, Kenney JS, Issekutz AC (1998). Chemokine production and adhesion molecule expression by neural cells exposed to IL-1, TNF[alpha] and interferont. *Life Sciences* **63**(21)**:** 1939-1952.

Chung YC, Kim SR, Jin BK (2010). Paroxetine Prevents Loss of Nigrostriatal Dopaminergic Neurons by Inhibiting Brain Inflammation and Oxidative Stress in an Experimental Model of Parkinson's Disease. *The Journal of Immunology* **185**(2)**:** 1230-1237.

Cicchetti F, Brownell AL, Williams K, Chen YI, Livni E, Isacson O (2002). Neuroinflammation of the nigrostriatal pathway during progressive 6-OHDA dopamine degeneration in rats monitored by immunohistochemistry and PET imaging. *Eur J Neurosci* **15**(6)**:** 991-998.

Ciechanover A (1998). The ubiquitin-proteasome pathway: on protein death and cell life. *EMBO J* **17**(24)**:** 7151-7160.

Clarke DJ, Branton RL (2002). A role for tumor necrosis factor alpha in death of dopaminergic neurons following neural transplantation. *Exp Neurol* **176**(1)**:** 154-162.

Cleeter MW, Cooper JM, Schapira AH (1992). Irreversible inhibition of mitochondrial complex I by 1-methyl-4-phenylpyridinium: evidence for free radical involvement. *J Neurochem* **58**(2)**:** 786-789.

Cohen G, Spina MB (1989). Deprenyl suppresses the oxidant stress associated with increased dopamine turnover. *Ann Neurol* **26**(5)**:** 689-690.

Conrad P, Rothman BL, Kelley KA, Blue ML (1992). Mechanism of peripheral T cell activation by coengagement of CD44 and CD2. *J Immunol* **149**(6)**:** 1833-1839.

Cordato DJ, Chan DK (2004). Genetics and Parkinson's disease. *Journal of clinical neuroscience : official journal of the Neurosurgical Society of Australasia* **11**(2)**:** 119-123.

Crawford HC, Matrisian LM, Liaw L (1998). Distinct Roles of Osteopontin in Host Defense Activity and Tumor Survival during Squamous Cell Carcinoma Progression in Vivo. *Cancer Res* **58**(22)**:** 5206-5215.

Cuervo AM, Stefanis L, Fredenburg R, Lansbury PT, Sulzer D (2004). Impaired degradation of mutant alpha-synuclein by chaperone-mediated autophagy. *Science* **305**(5688)**:** 1292-1295.

Czlonkowska A, Kohutnicka M, Kurkowska-Jastrzebska I, Czlonkowski A (1996). Microglial reaction in MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) induced Parkinson's disease mice model. *Neurodegeneration* **5**(2)**:** 137-143.

Dagda RK, Cherra SJ, 3rd, Kulich SM, Tandon A, Park D, Chu CT (2009). Loss of PINK1 function promotes mitophagy through effects on oxidative stress and mitochondrial fission. *J Biol Chem* **284**(20)**:** 13843-13855.

Dalia A, Neff NH, Hadjiconstantinou M (1993). GM1 ganglioside improves dopaminergic markers of rat mesencephalic cultures treated with MPP+. *J Neurosci* **13**(7)**:** 3104-3111.

Datki Z, Juhász A, Gálfi M, Soós K, Papp R, Zádori D, *et al.* (2003). Method for measuring neurotoxicity of aggregating polypeptides with the MTT assay on differentiated neuroblastoma cells. *Brain Research Bulletin* **62**(3)**:** 223-229.

de la Fuente-Fernandez R, Schulzer M, Mak E, Kishore A, Calne DB (1998). The role of the Lewy body in idiopathic Parkinsonism. *Parkinsonism Relat Disord* **4**(2)**:** 73-77.

Dehmer T, Lindenau J, Haid S, Dichgans J, Schulz JB (2000). Deficiency of inducible nitric oxide synthase protects against MPTP toxicity in vivo. *J Neurochem* **74**(5)**:** 2213-2216.

Deierborg T, Soulet D, Roybon L, Hall V, Brundin P (2008). Emerging restorative treatments for Parkinson's disease. *Prog Neurobiol* **85**(4)**:** 407-432.

Dekkers BG, Bos IS, Gosens R, Halayko AJ, Zaagsma J, Meurs H (2010). The integrin-blocking peptide RGDS inhibits airway smooth muscle remodeling in a guinea pig model of allergic asthma. *American journal of respiratory and critical care medicine* **181**(6)**:** 556-565.

Del Río P, Irmler M, Arango-González B, Favor J, Bobe C, Bartsch U, *et al.* (2011). GDNF-induced osteopontin from Müller glial cells promotes photoreceptor survival in the Pde6brd1 mouse model of retinal degeneration. *Glia* **59**(5)**:** 821-832.

DelleDonne A, Klos KJ, Fujishiro H, Ahmed Z, Parisi JE, Josephs KA, *et al.* (2008). Incidental Lewy body disease and preclinical Parkinson disease. *Arch Neurol* **65**(8)**:** 1074-1080.

den HJW, Bethlem J (1960). The distribution of Lewy bodies in the central and autonomic nervous systems in idiopathic paralysis agitans. *J Neurol Neurosurg Psychiatry* **23:** 283-290.

Denda S, Reichardt LF, Muller U (1998). Identification of Osteopontin as a Novel Ligand for the Integrin alpha 8beta 1 and Potential Roles for This Integrin-Ligand Interaction in Kidney Morphogenesis Vol. 9, pp 1425-1435.

Denhardt DT, Chambers AF (1994). Overcoming obstacles to metastasis - defenses against host defenses: Osteopontin (OPN) as a shield against attack by cytotoxic host cells. *Journal of cellular biochemistry* **56**(1)**:** 48-51.

Denhardt DT, Guo X (1993). Osteopontin: a protein with diverse functions. *FASEB J* **7**(15)**:** 1475-1482.

Denhardt DT, Lopez CA, Rollo EE, Hwang SM, An XR, Walther SE (1995). Osteopontin-induced modifications of cellular functions. *Annals of the New York Academy of Sciences* **760:** 127-142.

Denhardt DT, Noda M (1998). Osteopontin expression and function: role in bone remodeling. *Journal of cellular biochemistry. Supplement* **30-31:** 92-102.

Denhardt DT, Noda M, O'Regan AW, Pavlin D, Berman JS (2001). Osteopontin as a means to cope with environmental insults: regulation of inflammation, tissue remodeling, and cell survival. *J. Clin. Invest.* **107**(9)**:** 1055-1061.

Dennis J, Bennett JP, Jr. (2003). Interactions among nitric oxide and Bcl-family proteins after MPP+ exposure of SH-SY5Y neural cells I: MPP+ increases mitochondrial NO and Bax protein. *Journal of neuroscience research* **72**(1)**:** 76-88.

Dexter DT, Carter CJ, Wells FR, Javoy-Agid F, Agid Y, Lees A, *et al.* (1989a). Basal Lipid Peroxidation in Substantia Nigra Is Increased in Parkinson's Disease. *Journal of Neurochemistry* **52**(2)**:** 381-389.

Dexter DT, Sian J, Rose S, Hindmarsh JG, Mann VM, Cooper JM, *et al.* (1994). Indices of oxidative stress and mitochondrial function in individuals with incidental Lewy body disease. *Ann Neurol* **35**(1)**:** 38-44.

Dexter DT, Wells FR, Lees AJ, Agid F, Agid Y, Jenner P, *et al.* (1989b). Increased nigral iron content and alterations in other metal ions occurring in brain in Parkinson's disease. *J Neurochem* **52**(6)**:** 1830-1836.

Di Monte DA, Lavasani M, Manning-Bog AB (2002). Environmental factors in Parkinson's disease. *Neurotoxicology* **23**(4-5)**:** 487-502.

Doyle KP, Yang T, Lessov NS, Ciesielski TM, Stevens SL, Simon RP, *et al.* (2008). Nasal administration of osteopontin peptide mimetics confers neuroprotection in stroke. *J Cereb Blood Flow Metab* **28**(6)**:** 1235-1248.

Dringen R (2005). Oxidative and Antioxidative Potential of Brain Microglial Cells. *Antioxidants & Redox Signaling* **7**(9-10)**:** 1223-1233.

Du Y, Ma Z, Lin S, Dodel RC, Gao F, Bales KR, *et al.* (2001). Minocycline prevents nigrostriatal dopaminergic neurodegeneration in the MPTP model of Parkinson's disease. *Proc Natl Acad Sci U S A* **98**(25)**:** 14669-14674.

Dutta G, Zhang P, Liu B (2008). The lipopolysaccharide Parkinson's disease animal model: mechanistic studies and drug discovery. *Fundam Clin Pharmacol* **22**(5)**:** 453-464.

Earle KM (1968). Studies on Parkinson's disease including x-ray fluorescent spectroscopy of formalin fixed brain tissue. *J Neuropathol Exp Neurol* **27**(1)**:** 1-14.

Echtay KS, Winkler E, Klingenberg M (2000). Coenzyme Q is an obligatory cofactor for uncoupling protein function. *Nature* **408**(6812)**:** 609-613.

Eddleston M, Mucke L (1993). Molecular profile of reactive astrocytes--implications for their role in neurologic disease. *Neuroscience* **54**(1)**:** 15-36.

Ek-Rylander B, Flores M, Wendel M, Heinegard D, Andersson G (1994). Dephosphorylation of osteopontin and bone sialoprotein by osteoclastic tartrate-resistant acid phosphatase. Modulation of osteoclast adhesion in vitro. *J Biol Chem* **269**(21)**:** 14853-14856.

Ekström PAR, Mayer U, Panjwani A, Pountney D, Pizzey J, Tonge DA (2003). Involvement of [alpha]7[beta]1 integrin in the conditioning-lesion effect on sensory axon regeneration. *Molecular and Cellular Neuroscience* **22**(3)**:** 383-395.

Ellison JA, Barone FC, Feuerstein GZ (1999). Matrix Remodeling after Stroke: De Novo Expression of Matrix Proteins and Integrin Receptors Vol. 890, pp 204-222.

Ellison JA, Velier JJ, Spera P, Jonak ZL, Wang X, Barone FC, *et al.* (1998). Osteopontin and its integrin receptor alpha(v)beta3 are upregulated during formation of the glial scar after focal stroke. *Stroke* **29**(8)**:** 1698-1706; discussion 1707.

Ernster L, Dallner G (1995). Biochemical, physiological and medical aspects of ubiquinone function. *Biochim Biophys Acta* **1271**(1)**:** 195-204.

EstÉVez AG, JordÁN J (2002). Nitric Oxide and Superoxide, a Deadly Cocktail. *Annals of the New York Academy of Sciences* **962**(1)**:** 207-211.

Fall CP, Bennett JP, Jr. (1999). Characterization and time course of MPP+ -induced apoptosis in human SH-SY5Y neuroblastoma cells. *Journal of neuroscience research* **55**(5)**:** 620-628.

Farrer M, Kachergus J, Forno L, Lincoln S, Wang DS, Hulihan M, *et al.* (2004). Comparison of kindreds with parkinsonism and alpha-synuclein genomic multiplications. *Ann Neurol* **55**(2)**:** 174-179.

Fearnley JM, Lees AJ (1991). Ageing and Parkinson's disease: substantia nigra regional selectivity. *Brain* **114 (Pt 5):** 2283-2301.

Feng B, Rollo EE, Denhardt DT (1995). Osteopontin (OPN) may facilitate metastasis by protecting cells from macrophage NO-mediated cytotoxicity: evidence from cell lines down-regulated for OPN expression by a targeted ribozyme. *Clinical and Experimental Metastasis* **13**(6)**:** 453-462.

Feng F, Rittling SR (2000). Mammary tumor development in MMTV-c-myc/MMTV-v-Ha-ras transgenic mice is unaffected by osteopontin deficiency. *Breast Cancer Research and Treatment* **63**(1)**:** 71-79.

Ferrante RJ, Andreassen OA, Dedeoglu A, Ferrante KL, Jenkins BG, Hersch SM, *et al.* (2002). Therapeutic effects of coenzyme Q10 and remacemide in transgenic mouse models of Huntington's disease. *J Neurosci* **22**(5)**:** 1592-1599.

Fisher LW, Torchia DA, Fohr B, Young MF, Fedarko NS (2001). Flexible Structures of SIBLING Proteins, Bone Sialoprotein, and Osteopontin. *Biochemical and Biophysical Research Communications* **280**(2)**:** 460-465.

Floor E, Wetzel MG (1998). Increased Protein Oxidation in Human Substantia Nigra Pars Compacta in Comparison with Basal Ganglia and Prefrontal Cortex Measured with an Improved Dinitrophenylhydrazine Assay. *Journal of Neurochemistry* **70**(1)**:** 268-275.

Forno LS, DeLanney LE, Irwin I, Di Monte D, Langston JW (1992). Astrocytes and Parkinson's disease. *Prog Brain Res* **94:** 429-436.

Fox SB, Gatter KC, Jackson DG, Screaton GR, Bell MV, Bell JI, *et al.* (1993). CD44 and cancer screening. *Lancet* **342**(8870)**:** 548-549.

Funahashi H, Takeyama H, Sawai H, Furuta A, Sato M, Okada Y, *et al.* (2003). Alteration of integrin expression by glial cell line-derived neurotrophic factor (GDNF) in human pancreatic cancer cells. *Pancreas* **27**(2)**:** 190-196.

Furger KA, Menon RK, Tuck AB, Bramwell VHC, Chambers AF (2001). The Functional and Clinical Roles of Osteopontin in Cancer and Metastasis. *Current Molecular Medicine* **1:** 621-632.

Fuxe K, Agnati LF, Tinner B, Forsberg N, McCourt P, Gustafson S (1996). Evidence for receptors for hyaluronan in discrete nerve cell populations of the brain. *Brain Res* **736**(1-2)**:** 329-337.

Gao H-M, Liu B, Zhang W, Hong J-S (2003). Novel anti-inflammatory therapy for Parkinson's disease. *Trends in pharmacological sciences* **24**(8)**:** 395-401.

Gao HM, Jiang J, Wilson B, Zhang W, Hong JS, Liu B (2002). Microglial activation-mediated delayed and progressive degeneration of rat nigral dopaminergic neurons: relevance to Parkinson's disease. *J Neurochem* **81**(6)**:** 1285-1297.

Gardiner NJ, Moffatt S, Fernyhough P, Humphries MJ, Streuli CH, Tomlinson DR (2007). Preconditioning injury-induced neurite outgrowth of adult rat sensory neurons on fibronectin is mediated by mobilisation of axonal [alpha]5 integrin. *Molecular and Cellular Neuroscience* **35**(2)**:** 249-260.

Gardner HA, Berse B, Senger DR (1994). Specific reduction in osteopontin synthesis by antisense RNA inhibits the tumorigenicity of transformed Rat1 fibroblasts. *Oncogene* **9**(8)**:** 2321-2326.

Gasser T, Muller-Myhsok B, Wszolek ZK, Oehlmann R, Calne DB, Bonifati V, *et al.* (1998). A susceptibility locus for Parkinson's disease maps to chromosome 2p13. *Nat Genet* **18**(3)**:** 262-265.

Gatto NM, Cockburn M, Bronstein J, Manthripragada AD, Ritz B (2009). Well-water consumption and Parkinson's disease in rural California. *Environmental health perspectives* **117**(12)**:** 1912-1918.

Gautier CA, Kitada T, Shen J (2008). Loss of PINK1 causes mitochondrial functional defects and increased sensitivity to oxidative stress. *Proc Natl Acad Sci U S A* **105**(32)**:** 11364-11369.

Gayle DA, Ling Z, Tong C, Landers T, Lipton JW, Carvey PM (2002). Lipopolysaccharide (LPS)-induced dopamine cell loss in culture: roles of tumor necrosis factor-alpha, interleukin-1beta, and nitric oxide. *Brain Res Dev Brain Res* **133**(1)**:** 27-35.

Gélinas S, Martinoli M-G (2002). Neuroprotective effect of estradiol and phytoestrogens on MPP+-induced cytotoxicity in neuronal PC12 cells. *Journal of neuroscience research* **70**(1)**:** 90-96.

Gericke A, Qin C, Spevak L, Fujimoto Y, Butler WT, Sørensen ES, *et al.* (2005). Importance of Phosphorylation for Osteopontin Regulation of Biomineralization. *Calcified Tissue International* **77**(1)**:** 45-54.

Giachelli CM, Liaw L, Murry CE, Schwartz SM, Almeida M (1995). Osteopontin expression in cardiovascular diseases. *Annals of the New York Academy of Sciences* **760:** 109-126.

Giachelli CM, Lombardi D, Johnson RJ, Murry CE, Almeida M (1998). Evidence for a role of osteopontin in macrophage infiltration in response to pathological stimuli in vivo. *Am J Pathol* **152**(2)**:** 353-358.

Giasson BI, Duda JE, Murray IV, Chen Q, Souza JM, Hurtig HI, *et al.* (2000). Oxidative damage linked to neurodegeneration by selective alpha-synuclein nitration in synucleinopathy lesions. *Science (New York, N.Y* **290**(5493)**:** 985-989.

Gibson RM, Craig SE, Heenan L, Tournier C, Humphries MJ (2005). Activation of integrin [alpha]5[beta]1 delays apoptosis of Ntera2 neuronal cells. *Molecular and Cellular Neuroscience* **28**(3)**:** 588-598.

Gilbert EA, Edwards RJ, Boobis AR, Rose S, Jenner P (2003). Differential expression of cytochrome P450 enzymes in cultured and intact foetal rat ventral mesencephalon. *J Neural Transm* **110**(10)**:** 1091-1101.

Gill SS, Patel NK, Hotton GR, O'Sullivan K, McCarter R, Bunnage M, *et al.* (2003). Direct brain infusion of glial cell line-derived neurotrophic factor in Parkinson disease. *Nat Med* **9**(5)**:** 589-595.

Gombotz WR, Pettit DK (1995). Biodegradable polymers for protein and peptide drug delivery. *Bioconjugate chemistry* **6**(4)**:** 332-351.

Gomez C, Reiriz J, Pique M, Gil J, Ferrer I, Ambrosio S (2001). Low concentrations of 1-methyl-4-phenylpyridinium ion induce caspase-mediated apoptosis in human SH-SY5Y neuroblastoma cells. *Journal of neuroscience research* **63**(5)**:** 421-428.

Good PF, Hsu A, Werner P, Perl DP, Olanow CW (1998). Protein nitration in Parkinson's disease. *J Neuropathol Exp Neurol* **57**(4)**:** 338-342.

Goralski KB, Renton KW (2004). Brain inflammation enhances 1-methyl-4-phenylpyridinium-evoked neurotoxicity in rats. *Toxicol Appl Pharmacol* **196**(3)**:** 381-389.

Gordon MN, Schreier WA, Ou X, Holcomb LA, Morgan DG (1997). Exaggerated astrocyte reactivity after nigrostriatal deafferentation in the aged rat. *J Comp Neurol* **388**(1)**:** 106-119.

Gorell JM, Johnson CC, Rybicki BA, Peterson EL, Richardson RJ (1998). The risk of Parkinson's disease with exposure to pesticides, farming, well water, and rural living. *Neurology* **50**(5)**:** 1346-1350.

Gorski J (1992). Acidic phosphoproteins from bone matrix: A structural rationalization of their role in biomineralization. *Calcified Tissue International* **50**(5)**:** 391-396.

Graessmann M, Berg B, Fuchs B, Klein A, Graessmann A (2006). Chemotherapy resistance of mouse WAP-SVT//t breast cancer cells is mediated by osteopontin, inhibiting apoptosis downstream of caspase-3. *Oncogene* **26**(20)**:** 2840-2850.

Graham DG, TIFFANY SM, BELL WR, GUTKNECHT WF (1978). Autoxidation versus Covalent Binding of Quinones as the Mechanism of Toxicity of Dopamine, 6-Hydroxydopamine, and Related Compounds toward C1300 Neuroblastoma Cells in Vitro. *Molecular Pharmacology* **14**(4)**:** 644-653.

Grammatopoulos TN, Jones SM, Ahmadi FA, Hoover BR, Snell LD, Skoch J, *et al.* (2007). Angiotensin type 1 receptor antagonist losartan, reduces MPTP-induced degeneration of dopaminergic neurons in substantia nigra. *Mol Neurodegener* **2:** 1.

Greenamyre JT, Betarbet R, Sherer TB (2003). The rotenone model of Parkinson's disease: genes, environment and mitochondria. *Parkinsonism Relat Disord* **9 Suppl 2:** S59-64.

Greene LA, Aletta JM, Rukenstein A, Green SH (1987). PC12 pheochromocytoma cells: culture, nerve growth factor treatment, and experimental exploitation. *Methods in enzymology* **147:** 207-216.

Gross N, Beck D, Beretta C, Jackson D, Perruisseau G (1995). CB44 expression and modulation on human neuroblastoma tumours and cell lines. *European Journal of Cancer* **31**(4)**:** 471-475.

Gross N, Beretta C, Peruisseau G, Jackson D, Simmons D, Beck D (1994). CD44H Expression by Human Neuroblastoma Cells: Relation to MYCN Amplification and Lineage Differentiation Vol. 54, pp 4238-4242.

Gu J, Taniguchi N (2004). Regulation of integrin functions by N-glycans. *Glycoconj J* **21**(1-2)**:** 9-15.

Gu M, Cooper JM, Taanman JW, Schapira AH (1998). Mitochondrial DNA transmission of the mitochondrial defect in Parkinson's disease. *Ann Neurol* **44**(2)**:** 177-186.

Gunthert U, Hofmann M, Rudy W, Reber S, Zoller M, Haussmann I, *et al.* (1991). A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. *Cell* **65**(1)**:** 13-24.

Guo H, Cai CQ, Schroeder RA, Kuo PC (2001). Osteopontin Is a Negative Feedback Regulator of Nitric Oxide Synthesis in Murine Macrophages Vol. 166, pp 1079-1086.

Haas RH, Nasirian F, Nakano K, Ward D, Pay M, Hill R, *et al.* (1995). Low platelet mitochondrial complex I and complex II/III activity in early untreated Parkinson's disease. *Ann Neurol* **37**(6)**:** 714-722.

Haegel H, Tolg C, Hofmann M, Ceredig R (1993). Activated mouse astrocytes and T cells express similar CD44 variants. Role of CD44 in astrocyte/T cell binding. *J Cell Biol* **122**(5)**:** 1067-1077.

Halkias IA, Haq I, Huang Z, Fernandez HH (2007). When should levodopa therapy be initiated in patients with Parkinson's disease? *Drugs Aging* **24**(4)**:** 261-273.

Halliday G, Herrero MT, Murphy K, McCann H, Ros-Bernal F, Barcia C, *et al.* (2009). No Lewy pathology in monkeys with over 10 years of severe MPTP Parkinsonism. *Movement Disorders* **24**(10)**:** 1519-1523.

Halliday GM, Li YW, Blumbergs PC, Joh TH, Cotton RGH, Howe PRC, *et al.* (1990). Neuropathology of immunohistochemically identified brainstem neurons in Parkinson's disease. *Annals of Neurology* **27**(4)**:** 373-385.

Hanagasi HA, Emre M (2005). Treatment of behavioural symptoms and dementia in Parkinson's disease. *Fundamental & Clinical Pharmacology* **19**(2)**:** 133-146.

Hardy J (2010). Genetic analysis of pathways to Parkinson disease. *Neuron* **68**(2)**:** 201-206.

Harkavyi A, Abuirmeileh A, Lever R, Kingsbury AE, Biggs CS, Whitton PS (2008). Glucagon-like peptide 1 receptor stimulation reverses key deficits in distinct rodent models of Parkinson's disease. *Journal of neuroinflammation* **5:** 19.

Harkavyi A, Whitton PS (2010). Glucagon-like peptide 1 receptor stimulation as a means of neuroprotection. *British journal of pharmacology* **159**(3)**:** 495-501.

Hartley A, Stone JM, Heron C, Cooper JM, Schapira AHV (1994). Complex I inhibitors induce dose-dependent apoptosis in PC12 cells: Relevance to Parkinson's disease. *Journal of Neurochemistry* **63**(5)**:** 1987-1990.

Hartmann A, Hunot S, Michel PP, Muriel MP, Vyas S, Faucheux BA, *et al.* (2000). Caspase-3: A vulnerability factor and final effector in apoptotic death of dopaminergic neurons in Parkinson's disease. *Proc Natl Acad Sci U S A* **97**(6)**:** 2875-2880.

Hasegawa E, Takeshige K, Oishi T, Murai Y, Minakami S (1990a). 1-Methyl-4-phenylpyridinium (MPP+) induces NADH-dependent superoxide formation and enhances NADH-dependent lipid peroxidation in bovine heart submitochondrial particles. *Biochem Biophys Res Commun* **170**(3)**:** 1049-1055.

Hasegawa E, Takeshige K, Oishi T, Murai Y, Minakami S (1990b). 1-Methyl-4-phenylpyridinium (MPP+) induces NADH-dependent superoxide formation and enhances NADH-dependent lipid peroxidation in bovine heart submitochondrial particles. *Biochemical and Biophysical Research Communications* **170**(3)**:** 1049-1055.

Hashimoto M, Sun D, Rittling SR, Denhardt DT, Young W (2007). Osteopontin-deficient mice exhibit less inflammation, greater tissue damage, and impaired locomotor recovery from spinal cord injury compared with wild-type controls. *J Neurosci* **27**(13)**:** 3603-3611.

Hattori N, Tanaka M, Ozawa T, Mizuno Y (1991). Immunohistochemical studies on complexes I, II, III, and IV of mitochondria in Parkinson's disease. *Ann Neurol* **30**(4)**:** 563-571.

Hauser RA, Zesiewicz TA (2006). Clinical trials aimed at detecting neuroprotection in Parkinson's disease. *Neurology* **66**(10 Suppl 4)**:** S58-68.

He Y, Appel S, Le W (2001). Minocycline inhibits microglial activation and protects nigral cells after 6-hydroxydopamine injection into mouse striatum. *Brain Research* **909**(1-2)**:** 187-193.

He Y, Lee T, Leong SK (2000). 6-Hydroxydopamine induced apoptosis of dopaminergic cells in the rat substantia nigra. *Brain Res* **858**(1)**:** 163-166.

Henze C, Hartmann A, Lescot T, Hirsch EC, Michel PP (2005). Proliferation of microglial cells induced by 1-methyl-4-phenylpyridinium in mesencephalic cultures results from an astrocyte-dependent mechanism: role of granulocyte macrophage colony-stimulating factor. *J Neurochem* **95**(4)**:** 1069-1077.

Hernan MA, Logroscino G, Garcia Rodriguez LA (2006). Nonsteroidal anti-inflammatory drugs and the incidence of Parkinson disease. *Neurology* **66**(7)**:** 1097-1099.

Herrera AJ, Castano A, Venero JL, Cano J, Machado A (2000). The single intranigral injection of LPS as a new model for studying the selective effects of inflammatory reactions on dopaminergic system. *Neurobiol Dis* **7**(4)**:** 429-447.

Hirsch EC (2000). Glial cells and Parkinson's disease. *J Neurol* **247 Suppl 2:** II58-62.

Hirsch EC, Breidert T, Rousselet E, Hunot S, Hartmann A, Michel PP (2003). The role of glial reaction and inflammation in Parkinson's disease. *Annals of the New York Academy of Sciences* **991:** 214-228.

Hochstrasser M (1996). Ubiquitin-dependent protein degradation. *Annu Rev Genet* **30:** 405-439.

Hoffer BJ, Hoffman A, Bowenkamp K, Huettl P, Hudson J, Martin D, *et al.* (1994). Glial cell line-derived neurotrophic factor reverses toxin-induced injury to midbrain dopaminergic neurons in vivo. *Neurosci Lett* **182**(1)**:** 107-111.

Homandberg GA, Hui F (1994). ARG-Gly-AsP-Ser Peptide Analogs Suppress Cartilage Chondrolytic Activities of Integrin-Binding and Nonbinding Fibronectin Fragments. *Archives of Biochemistry and Biophysics* **310**(1)**:** 40-48.

Horger BA, Nishimura MC, Armanini MP, Wang L-C, Poulsen KT, Rosenblad C, *et al.* (1998). Neurturin Exerts Potent Actions on Survival and Function of Midbrain Dopaminergic Neurons. *The Journal of Neuroscience* **18**(13)**:** 4929-4937.

Hu DD, Lin ECK, Kovach NL, Hoyer JR, Smith JW (1995). A Biochemical Characterization of the Binding of Osteopontin to Integrins alpha(v)beta(1) and alpha(v)beta(5) Vol. 270, pp 26232-26238.

Hudkins KL, Giachelli CM, Cui Y, Couser WG, Johnson RJ, Alpers CE (1999). Osteopontin expression in fetal and mature human kidney. *J Am Soc Nephrol* **10**(3)**:** 444-457.

Huet S, Groux H, Caillou B, Valentin H, Prieur AM, Bernard A (1989). CD44 contributes to T cell activation. *J Immunol* **143**(3)**:** 798-801.

Hunot S, Boissiere F, Faucheux B, Brugg B, Mouatt-Prigent A, Agid Y, *et al.* (1996). Nitric oxide synthase and neuronal vulnerability in parkinson's disease. *Neuroscience* **72**(2)**:** 355-363.

Hunot S, Dugas N, Faucheux B, Hartmann A, Tardieu M, Debre P, *et al.* (1999). FcepsilonRII/CD23 is expressed in Parkinson's disease and induces, in vitro, production of nitric oxide and tumor necrosis factor-alpha in glial cells. *J Neurosci* **19**(9)**:** 3440-3447.

Hur EM, Youssef S, Haws ME, Zhang SY, Sobel RA, Steinman L (2007). Osteopontin-induced relapse and progression of autoimmune brain disease through enhanced survival of activated T cells. *Nat Immunol* **8**(1)**:** 74-83.

Hwang SM, Lopez CA, Heck DE, Gardner CR, Laskin DL, Laskin JD, *et al.* (1994a). Osteopontin inhibits induction of nitric oxide synthase gene expression by inflammatory mediators in mouse kidney epithelial cells Vol. 269, pp 711-715.

Hwang SM, Wilson PD, Laskin JD, Denhardt DT (1994b). Age and development-related changes in osteopontin and nitric oxide synthase mRNA levels in human kidney proximal tubule epithelial cells: contrasting responses to hypoxia and reoxygenation. *J Cell Physiol* **160**(1)**:** 61-68.

Iczkiewicz J, Broom L, Cooper JD, Wong AM, Rose S, Jenner P (2010). The RGD-containing peptide fragment of osteopontin protects tyrosine hydroxylase positive cells against toxic insult in primary ventral mesencephalic cultures and in the rat substantia nigra. *J Neurochem* **114**(6)**:** 1792-1804.

Iczkiewicz J, Jackson MJ, Smith LA, Rose S, Jenner P (2006). Osteopontin expression in substantia nigra in MPTP-treated primates and in Parkinson's disease. *Brain Research* **1118**(1)**:** 239-250.

Iczkiewicz J, Rose S, Jenner P (2005). Increased osteopontin expression following intranigral lipopolysaccharide injection in the rat Vol. 21, pp 1911-1920.

Iczkiewicz J, Rose S, Jenner P (2004). Osteopontin (Eta-1) is present in the rat basal ganglia. *Molecular Brain Research* **132**(1)**:** 64-72.

Imlay JA (2003). Pathways of oxidative damage. *Annual review of microbiology* **57:** 395-418.

investigators NN-P (2008). A pilot clinical trial of creatine and minocycline in early Parkinson disease: 18-month results. *Clinical neuropharmacology* **31**(3)**:** 141-150.

Inzelberg R, Jankovic J (2007). Are Parkinson disease patients protected from some but not all cancers? *Neurology* **69**(15)**:** 1542-1550.

Iravani MM, Kashefi K, Mander P, Rose S, Jenner P (2002). Involvement of inducible nitric oxide synthase in inflammation-induced dopaminergic neurodegeneration. *Neuroscience* **110**(1)**:** 49-58.

Iravani MM, Leung CC, Sadeghian M, Haddon CO, Rose S, Jenner P (2005). The acute and the long-term effects of nigral lipopolysaccharide administration on dopaminergic dysfunction and glial cell activation. *Eur J Neurosci* **22**(2)**:** 317-330.

Isacke CM, Yarwood H (2002). The hyaluronan receptor, CD44. *Int J Biochem Cell Biol* **34**(7)**:** 718-721.

Ishida Y, Nagai A, Kobayashi S, Kim SU (2006). Upregulation of Protease-Activated Receptor-1 in Astrocytes in Parkinson Disease: Astrocyte-Mediated Neuroprotection Through Increased Levels of Glutathione Peroxidase. *Journal of Neuropathology & Experimental Neurology* **65**(1)**:** 66-77 10.1097/1001.jnen.0000195941.0000148033.eb.

Jalkanen ST, Bargatze RF, Herron LR, Butcher EC (1986). A lymphoid cell surface glycoprotein involved in endothelial cell recognition and lymphocyte homing in man. *Eur J Immunol* **16**(10)**:** 1195-1202.

Janetzky B, Hauck S, Youdim MB, Riederer P, Jellinger K, Pantucek F, *et al.* (1994). Unaltered aconitase activity, but decreased complex I activity in substantia nigra pars compacta of patients with Parkinson's disease. *Neurosci Lett* **169**(1-2)**:** 126-128.

Jankovic J (2008). Parkinson's disease: clinical features and diagnosis. *J Neurol Neurosurg Psychiatry* **79**(4)**:** 368-376.

Jankovic J (2005). Searching for a relationship between manganese and welding and Parkinson's disease. *Neurology* **64**(12)**:** 2021-2028.

Jansson M, Panoutsakopoulou V, Baker J, Klein L, Cantor H (2002). Cutting edge: Attenuated experimental autoimmune encephalomyelitis in eta-1/osteopontin-deficient mice. *J Immunol* **168**(5)**:** 2096-2099.

Javoy-Agid F, Ruberg M, Taquet H, Bokobza B, Agid Y, Gaspar P, *et al.* (1984). Biochemical neuropathology of Parkinson's disease. *Adv Neurol* **40:** 189-198.

Jellinger K (2008). A critical reappraisal of current staging of Lewy-related pathology in human brain. *Acta Neuropathologica* **116**(1)**:** 1-16.

Jellinger KA (2000). Cell death mechanisms in Parkinson's disease. *J Neural Transm* **107**(1)**:** 1-29.

Jin JK, Na YJ, Moon C, Kim H, Ahn M, Kim YS, *et al.* (2006). Increased expression of osteopontin in the brain with scrapie infection. *Brain Res* **1072**(1)**:** 227-233.

Joglar B, Rodriguez-Pallares J, Rodriguez-Perez AI, Rey P, Guerra MJ, Labandeira-Garcia JL (2009). The inflammatory response in the MPTP model of Parkinson's disease is mediated by brain angiotensin: relevance to progression of the disease. *J Neurochem* **109**(2)**:** 656-669.

Johansson S, Stromberg I (2003). Fetal lateral ganglionic eminence attracts one of two morphologically different types of tyrosine hydroxylase-positive nerve fibers formed by cultured ventral mesencephalon. *Cell Transplant* **12**(3)**:** 243-255.

Johnston LC, Su X, Maguire-Zeiss K, Horovitz K, Ankoudinova I, Guschin D, *et al.* (2008). Human Interleukin-10 Gene Transfer Is Protective in a Rat Model of Parkinson's Disease. *Mol Ther* **16**(8)**:** 1392-1399.

Jono S, Peinado C, Giachelli CM (2000). Phosphorylation of osteopontin is required for inhibition of vascular smooth muscle cell calcification. *J Biol Chem* **275**(26)**:** 20197-20203.

Kasugai S, Zhang Q, Overall CM, Wrana JL, Butler WT, Sodek J (1991). Differential regulation of the 55 and 44 kDa forms of secreted phosphoprotein 1 (SPP-1, osteopontin) in normal and transformed rat bone cells by osteotropic hormones, growth factors and a tumor promoter. *Bone and Mineral* **13**(3)**:** 235-250.

Katagiri YU, Sleeman J, Fujii H, Herrlich P, Hotta H, Tanaka K, *et al.* (1999). CD44 Variants but not CD44s Cooperate with {beta}1-containing Integrins to Permit Cells to Bind to Osteopontin Independently of Arginine-glycine-aspartic Acid, thereby Stimulating Cell Motility and Chemotaxis Vol. 59, pp 219-226.

Kawashima R, Mochida S, Matsui A, YouLuTuZ Y, Ishikawa K, Toshima K, *et al.* (1999). Expression of Osteopontin in Kupffer Cells and Hepatic Macrophages and Stellate Cells in Rat Liver after Carbon Tetrachloride Intoxication: A Possible Factor for Macrophage Migration into Hepatic Necrotic Areas. *Biochemical and Biophysical Research Communications* **256**(3)**:** 527-531.

Keeney PM, Xie J, Capaldi RA, Bennett JP, Jr. (2006). Parkinson's disease brain mitochondrial complex I has oxidatively damaged subunits and is functionally impaired and misassembled. *J Neurosci* **26**(19)**:** 5256-5264.

Kenchappa RS, Diwakar L, Annepu J, Ravindranath V (2004). Estrogen and neuroprotection: higher constitutive expression of glutaredoxin in female mice offers protection against MPTP-mediated neurodegeneration. *The FASEB Journal*.

Kennedy LJ, Moore K, Caulfield JL, Tannenbaum SR, Dedon PC (1997). Quantitation of 8-Oxoguanine and Strand Breaks Produced by Four Oxidizing Agents. *Chemical Research in Toxicology* **10**(4)**:** 386-392.

Keykhosravani M, Doherty-Kirby A, Zhang C, Brewer D, Goldberg HA, Hunter GK, *et al.* (2005). Comprehensive identification of post-translational modifications of rat bone osteopontin by mass spectrometry. *Biochemistry* **44**(18)**:** 6990-7003.

Khan SA, Lopez-Chua CA, Zhang J, Fisher LW, Sørensen ES, Denhardt DT (2002). Soluble osteopontin inhibits apoptosis of adherent endothelial cells deprived of growth factors Vol. 85, pp 728-736.

Kim HJ, Lee HJ, Jun JI, Oh Y, Choi SG, Kim H, *et al.* (2009). Intracellular cleavage of osteopontin by caspase-8 modulates hypoxia/reoxygenation cell death through p53. *Proc Natl Acad Sci U S A* **106**(36)**:** 15326-15331.

Kim MD, Cho HJ, Shin T (2004). Expression of osteopontin and its ligand, CD44, in the spinal cords of Lewis rats with experimental autoimmune encephalomyelitis. *J Neuroimmunol* **151**(1-2)**:** 78-84.

Kim RH, Smith PD, Aleyasin H, Hayley S, Mount MP, Pownall S, *et al.* (2005). Hypersensitivity of DJ-1-deficient mice to 1-methyl-4-phenyl-1,2,3,6-

tetrahydropyrindine (MPTP) and oxidative stress. *Proc Natl Acad Sci U S A* **102**(14)**:** 5215-5220.

Kim SY, Choi YS, Choi JS, Cha JH, Kim ON, Lee SB, *et al.* (2002). Osteopontin in kainic acid-induced microglial reactions in the rat brain. *Mol Cells* **13**(3)**:** 429-435.

King TD, Jope RS (2005). Inhibition of glycogen synthase kinase-3 protects cells from intrinsic but not extrinsic oxidative stress. *Neuroreport* **16**(6)**:** 597-601.

Kingsbury AE, Mardsen CD, Foster OJ (1998). DNA fragmentation in human substantia nigra: apoptosis or perimortem effect? *Mov Disord* **13**(6)**:** 877-884.

Kirik D, Rosenblad C, Burger C, Lundberg C, Johansen TE, Muzyczka N, *et al.* (2002). Parkinson-like neurodegeneration induced by targeted overexpression of alpha-synuclein in the nigrostriatal system. *J Neurosci* **22**(7)**:** 2780-2791.

Kitada T, Asakawa S, Hattori N, Matsumine H, Yamamura Y, Minoshima S, *et al.* (1998). Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature* **392**(6676)**:** 605-608.

Kitamura Y, Inden M, Miyamura A, Kakimura J, Taniguchi T, Shimohama S (2002). Possible involvement of both mitochondria- and endoplasmic reticulum-dependent caspase pathways in rotenone-induced apoptosis in human neuroblastoma SH-SY5Y cells. *Neurosci Lett* **333**(1)**:** 25-28.

Kleinman JG, Beshensky A, Worcester EM, Brown D (1995). Expression of osteopontin, a urinary inhibitor of stone mineral crystal growth, in rat kidney. *Kidney Int* **47**(6)**:** 1585-1596.

Klivenyi P, Andreassen OA, Ferrante RJ, Dedeoglu A, Mueller G, Lancelot E, *et al.* (2000). Mice deficient in cellular glutathione peroxidase show increased vulnerability to malonate, 3-nitropropionic acid, and 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. *J Neurosci* **20**(1)**:** 1-7.

Klöcker N, Bähr M (2001). AuthorBrain repair - new avenues to an eternal dream? *Trends in Neurosciences* **24**(1)**:** 3-4.

Kloss CUA, Bohatschek M, Kreutzberg GW, Raivich G (2001). Effect of Lipopolysaccharide on the Morphology and Integrin Immunoreactivity of Ramified Microglia in the Mouse Brain and in Cell Culture. *Experimental Neurology* **168**(1)**:** 32-46.

Knott C, Stern G, Kingsbury A, Welcher AA, Wilkin GP (2002). Elevated glial brain-derived neurotrophic factor in Parkinson's diseased nigra. *Parkinsonism & related disorders* **8**(5)**:** 329-341.

Knott C, Stern G, Wilkin GP (2000a). Inflammatory Regulators in Parkinson's Disease: iNOS, Lipocortin-1, and Cyclooxygenases-1 and -2. *Molecular and Cellular Neuroscience* **16**(6)**:** 724-739.

Knott C, Stern G, Wilkin GP (2000b). Inflammatory regulators in Parkinson's disease: iNOS, lipocortin-1, and cyclooxygenases-1 and -2. *Mol Cell Neurosci* **16**(6)**:** 724-739.

Kohutnicka M, Lewandowska E, Kurkowska-Jastrzebska I, Czlonkowski A, Czlonkowska A (1998). Microglial and astrocytic involvement in a murine model of Parkinson's disease induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *Immunopharmacology* **39**(3)**:** 167-180.

Kordower JH, Herzog CD, Dass B, Bakay RAE, Stansell J, Gasmi M, *et al.* (2006). Delivery of neurturin by AAV2 (CERE-120)-mediated gene transfer provides structural and functional neuroprotection and neurorestoration in MPTP-treated monkeys. *Annals of Neurology* **60**(6)**:** 706-715.

Kou W, Luchtman D, Song C (2008). Eicosapentaenoic acid (EPA) increases cell viability and expression of neurotrophin receptors in retinoic acid and brain-derived neurotrophic factor differentiated SH-SY5Y cells. *European Journal of Nutrition* **47**(2)**:** 104-113.

Krane SM, Glimcher MJ (1962). Transphosphorylation from nucleoside Di- and triphosphates by apatite crystals. *J Biol Chem* **237:** 2991-2998.

Kruger R, Kuhn W, Muller T, Woitalla D, Graeber M, Kosel S, *et al.* (1998). Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. *Nat Genet* **18**(2)**:** 106-108.

Kubota T, Zhang Q, Wrana JL, Ber R, Aubin JE, Butler WT, *et al.* (1989). Multiple forms of (secreted phosphoprotein, osteopontin) synthesized by normal and transformed rat bone cell populations: Regulation by TGF-[beta]. *Biochemical and Biophysical Research Communications* **162**(3)**:** 1453-1459.

Kurkowska-Jastrzebska I, Wronska A, Kohutnicka M, Czlonkowski A, Czlonkowska A (1999). The inflammatory reaction following 1-methyl-4-phenyl-1,2,3, 6-tetrahydropyridine intoxication in mouse. *Exp Neurol* **156**(1)**:** 50-61.

Lampe MA, Patarca R, Iregui MV, Cantor H (1991). Polyclonal B cell activation by the Eta-1 cytokine and the development of systemic autoimmune disease Vol. 147, pp 2902-2906.

Landfield PW, Thibault O, Mazzanti ML, Porter NM, Kerr DS (1992). Mechanisms of neuronal death in brain aging and Alzheimer's disease: role of endocrine-mediated calcium dyshomeostasis. *Journal of neurobiology* **23**(9)**:** 1247-1260.

Lang AE, Gill S, Patel NK, Lozano A, Nutt JG, Penn R, *et al.* (2006). Randomized controlled trial of intraputamenal glial cell line-derived neurotrophic factor infusion in Parkinson disease. *Ann Neurol* **59**(3)**:** 459-466.

Lang AE, Lozano AM (1998). Parkinson's Disease. *New England Journal of Medicine* **339**(15)**:** 1044-1053.

Langston J, Ballard P, Tetrud J, Irwin I (1983). Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. *Science* **219**(4587)**:** 979-980.

Langston JW, Forno LS (1978). The hypothalamus in Parkinson disease. *Ann Neurol* **3**(2)**:** 129-133.

Langston JW, Forno LS, Tetrud J, Reeves AG, Kaplan JA, Karluk D (1999). Evidence of active nerve cell degeneration in the substantia nigra of humans years after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine exposure. *Ann Neurol* **46**(4)**:** 598-605.

Lapchak PA, Miller PJ, Collins F, Jiao S (1997). Glial cell line-derived neurotrophic factor attenuates behavioural deficits and regulates nigrostriatal dopaminergic and peptidergic markers in 6-hydroxydopamine-lesioned adult rats: comparison of intraventricular and intranigral delivery. *Neuroscience* **78**(1)**:** 61-72.

Larsen JP, Boas J, Erdal JE (1999). Does selegiline modify the progression of early Parkinson's disease? Results from a five-year study. The Norwegian-Danish Study Group. *Eur J Neurol* **6**(5)**:** 539-547.

Lee J-L, Wang M-J, Sudhir P-R, Chen G-D, Chi C-W, Chen J-Y (2007). Osteopontin Promotes Integrin Activation through Outside-In and Inside-Out Mechanisms: OPN-CD44V Interaction Enhances Survival in Gastrointestinal Cancer Cells Vol. 67, pp 2089-2097.

Lees A (2005). Alternatives to levodopa in the initial treatment of early Parkinson's disease. *Drugs Aging* **22**(9)**:** 731-740.

Lees AJ (1995). Comparison of therapeutic effects and mortality data of levodopa and levodopa combined with selegiline in patients with early, mild Parkinson's disease. Parkinson's Disease Research Group of the United Kingdom. *BMJ* **311**(7020)**:** 1602-1607.

Lemons ML, Condic ML (2008). Integrin signaling is integral to regeneration. *Experimental Neurology* **209**(2)**:** 343-352.

Leroy E, Boyer R, Auburger G, Leube B, Ulm G, Mezey E, *et al.* (1998). The ubiquitin pathway in Parkinson's disease. *Nature* **395**(6701)**:** 451-452.

Levin S, Bucci TJ, Cohen SM, Fix AS, Hardisty JF, LeGrand EK, *et al.* (1999). The nomenclature of cell death: recommendations of an ad hoc Committee of the Society of Toxicologic Pathologists. *Toxicol Pathol* **27**(4)**:** 484-490.

Lewis PA, Greggio E, Beilina A, Jain S, Baker A, Cookson MR (2007). The R1441C mutation of LRRK2 disrupts GTP hydrolysis. *Biochem Biophys Res Commun* **357**(3)**:** 668-671.

Li Y, Hansotia T, Yusta B, Ris F, Halban PA, Drucker DJ (2003). Glucagon-like peptide-1 receptor signaling modulates beta cell apoptosis. *J Biol Chem* **278**(1)**:** 471-478.

Li Y, Perry T, Kindy MS, Harvey BK, Tweedie D, Holloway HW, *et al.* (2009). GLP-1 receptor stimulation preserves primary cortical and dopaminergic neurons in cellular and rodent models of stroke and Parkinsonism. *Proc Natl Acad Sci U S A* **106**(4)**:** 1285-1290.

Liaw L, Almeida M, Hart CE, Schwartz SM, Giachelli CM (1994). Osteopontin promotes vascular cell adhesion and spreading and is chemotactic for smooth muscle cells in vitro Vol. 74, pp 214-224.

Liaw L, Birk DE, Ballas CB, Whitsitt JS, Davidson JM, Hogan BL (1998a). Altered wound healing in mice lacking a functional osteopontin gene (spp1). *J Clin Invest* **101**(7)**:** 1468-1478.

Liaw L, Birk DE, Ballas CB, Whitsitt JS, Davidson JM, Hogan BLM (1998b). Altered Wound Healing in Mice Lacking a Functional Osteopontin Gene (spp1) Vol. 101, pp 1468-1478.

Liaw L, Skinner MP, Raines EW, Ross R, Cheresh DA, Schwartz SM, *et al.* (1995). The adhesive and migratory effects of osteopontin are mediated via distinct cell surface integrins. Role of alpha v beta 3 in smooth muscle cell migration to osteopontin in vitro. *J Clin Invest* **95**(2)**:** 713-724.

Liberatore GT, Jackson-Lewis V, Vukosavic S, Mandir AS, Vila M, McAuliffe WG, *et al.* (1999). Inducible nitric oxide synthase stimulates dopaminergic neurodegeneration in the MPTP model of Parkinson disease. *Nat Med* **5**(12)**:** 1403-1409.

Licinio J, Prolo P, McCann SM, Wong ML (1999). Brain iNOS: current understanding and clinical implications. *Mol Med Today* **5**(5)**:** 225-232.

Lieske JC, Leonard R, Toback FG (1995). Adhesion of calcium oxalate monohydrate crystals to renal epithelial cells is inhibited by specific anions. *Am J Physiol* **268**(4 Pt 2)**:** F604-612.

Lin D, Barnett M, Grauer L, Robben J, Jewell A, Takemoto L, *et al.* (2005). Expression of superoxide dismutase in whole lens prevents cataract formation. *Mol Vis* **11:** 853-858.

Lin WH, Higgins D, Pacheco M, Aletta J, Perini S, Marcucci KA, *et al.* (1993). Manganese induces spreading and process outgrowth in rat pheochromocytoma (PC12) cells. *Journal of neuroscience research* **34**(5)**:** 546-561.

Lin Y-H, Yang-Yen H-F (2001). The Osteopontin-CD44 Survival Signal Involves Activation of the Phosphatidylinositol 3-Kinase/Akt Signaling Pathway Vol. 276, pp 46024-46030.

Lin YH, Huang CJ, Chao JR, Chen ST, Lee SF, Yen JJ, *et al.* (2000). Coupling of osteopontin and its cell surface receptor CD44 to the cell survival response elicited

by interleukin-3 or granulocyte-macrophage colony-stimulating factor. *Mol Cell Biol* **20**(8)**:** 2734-2742.

Linazasoro G (2007). Classical Parkinson disease versus Parkinson complex – Reflections against staging and in favour of heterogeneity. *European Journal of Neurology* **14**(7)**:** 721-728.

Linnala A, Lehto VP, Virtanen I (1997). Neuronal differentiation in SH-SY5Y human neuroblastoma cells induces synthesis and secretion of tenascin and upregulation of alpha(v) integrin receptors. *Journal of neuroscience research* **49**(1)**:** 53-63.

Liou HH, Tsai MC, Chen CJ, Jeng JS, Chang YC, Chen SY, *et al.* (1997). Environmental risk factors and Parkinson's disease: a case-control study in Taiwan. *Neurology* **48**(6)**:** 1583-1588.

Litvan I, Bhatia KP, Burn DJ, Goetz CG, Lang AE, McKeith I, *et al.* (2003). SIC Task Force appraisal of clinical diagnostic criteria for parkinsonian disorders. *Movement Disorders* **18**(5)**:** 467-486.

Liu B, Jiang JW, Wilson BC, Du L, Yang SN, Wang JY, *et al.* (2000a). Systemic infusion of naloxone reduces degeneration of rat substantia nigral dopaminergic neurons induced by intranigral injection of lipopolysaccharide. *J Pharmacol Exp Ther* **295**(1)**:** 125-132.

Liu W, Kato M, Akhand AA, Hayakawa A, Suzuki H, Miyata T, *et al.* (2000b). 4-hydroxynonenal induces a cellular redox status-related activation of the caspase cascade for apoptotic cell death. *J Cell Sci* **113 (Pt 4):** 635-641.

Loh KP, Huang SH, De Silva R, Tan BK, Zhu YZ (2006). Oxidative stress: apoptosis in neuronal injury. *Curr Alzheimer Res* **3**(4)**:** 327-337.

Lohle M, Reichmann H (2010). Clinical neuroprotection in Parkinson's disease - still waiting for the breakthrough. *J Neurol Sci* **289**(1-2)**:** 104-114.

Lotharius J, Brundin P (2002). Impaired dopamine storage resulting from alpha-synuclein mutations may contribute to the pathogenesis of Parkinson's disease. *Hum Mol Genet* **11**(20)**:** 2395-2407.

Lotharius J, Dugan LL, O'Malley KL (1999). Distinct mechanisms underlie neurotoxin-mediated cell death in cultured dopaminergic neurons. *Journal of Neuroscience* **19**(4)**:** 1284-1293.

Luo ZD, Cizkova D (2000). The role of nitric oxide in nociception. *Current review of pain* **4**(6)**:** 459-466.

Maetzler W, Berg D, Schalamberidze N, Melms A, Schott K, Mueller JC, *et al.* (2007). Osteopontin is elevated in Parkinson's disease and its absence leads to reduced neurodegeneration in the MPTP model. *Neurobiol Dis* **25**(3)**:** 473-482.

Maetzler W, Michelis J, Tomiuk J, Melms A, Becker C, Gasser T, *et al.* (2009). A single-nucleotide polymorphism of the osteopontin gene may contribute to a susceptibility to Lewy body disease. *J Neural Transm* **116**(5)**:** 599-605.

Majno G, Joris I (1995). Apoptosis, oncosis, and necrosis. An overview of cell death. *Am J Pathol* **146**(1)**:** 3-15.

Mandel S, Grunblatt E, Riederer P, Gerlach M, Levites Y, Youdim MB (2003). Neuroprotective strategies in Parkinson's disease : an update on progress. *CNS drugs* **17**(10)**:** 729-762.

Mann VM, Cooper JM, Daniel SE, Srai K, Jenner P, Marsden CD, *et al.* (1994). Complex I, iron, and ferritin in Parkinson's disease substantia nigra. *Ann Neurol* **36**(6)**:** 876-881.

Marchetti B, Abbracchio MP (2005). To be or not to be (inflamed) - is that the question in anti-inflammatory drug therapy of neurodegenerative disorders? *Trends in pharmacological sciences* **26**(10)**:** 517-525.

Marcondes MC, Poling M, Watry DD, Hall D, Fox HS (2008). In vivo osteopontin-induced macrophage accumulation is dependent on CD44 expression. *Cell Immunol* **254**(1)**:** 56-62.

Mariani E, Polidori MC, Cherubini A, Mecocci P (2005). Oxidative stress in brain aging, neurodegenerative and vascular diseases: an overview. *J Chromatogr B Analyt Technol Biomed Life Sci* **827**(1)**:** 65-75.

Markey KA, Kondo S, Shenkman L, Goldstein M (1980). Purification and Characterization of Tyrosine Hydroxylase from a Clonal Pheochromocytoma Cell Line Vol. 17, pp 79-85.

Marks Jr WJ, Bartus RT, Siffert J, Davis CS, Lozano A, Boulis N, *et al.* (2010). Gene delivery of AAV2-neurturin for Parkinson's disease: a double-blind, randomised, controlled trial. *The Lancet Neurology* **9**(12)**:** 1164-1172.

Marks Jr WJ, Ostrem JL, Verhagen L, Starr PA, Larson PS, Bakay RAE, *et al.* (2008). Safety and tolerability of intraputaminal delivery of CERE-120 (adeno-associated virus serotype 2-neurturin) to patients with idiopathic Parkinson's disease: an open-label, phase I trial. *The Lancet Neurology* **7**(5)**:** 400-408.

Maroney AC, Glicksman MA, Basma AN, Walton KM, Knight E, Jr., Murphy CA, *et al.* (1998). Motoneuron apoptosis is blocked by CEP-1347 (KT 7515), a novel inhibitor of the JNK signaling pathway. *J Neurosci* **18**(1)**:** 104-111.

Maruyama W, Sango K, Iwasa K, Minami C, Dostert P, Kawai M, *et al.* (2000). Dopaminergic neurotoxins, 6,7-dihydroxy-1-(3', 4'-dihydroxybenzyl)-isoquinolines, cause different types of cell death in SH-SY5Y cells: apoptosis was induced by oxidized papaverolines and necrosis by reduced tetrahydropapaverolines. *Neurosci Lett* **291**(2)**:** 89-92.

McGeer PL, Itagaki S, Boyes BE, McGeer EG (1988). Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains. *Neurology* **38**(8)**:** 1285-1291.

McGeer PL, McGeer EG (1998). Glial cell reactions in neurodegenerative diseases: pathophysiology and therapeutic interventions. *Alzheimer Dis Assoc Disord* **12 Suppl 2:** S1-6.

McGeer PL, McGeer EG (2007). NSAIDs and Alzheimer disease: Epidemiological, animal model and clinical studies. *Neurobiology of aging* **28**(5)**:** 639-647.

McMahon A, Wong BS, Iacopino AM, Ng MC, Chi S, German DC (1998). Calbindin-D28k buffers intracellular calcium and promotes resistance to degeneration in PC12 cells. *Molecular Brain Research* **54**(1)**:** 56-63.

McNaught KS, Jenner P (1999a). Altered glial function causes neuronal death and increases neuronal susceptibility to 1-methyl-4-phenylpyridinium- and 6-hydroxydopamine-induced toxicity in astrocytic/ventral mesencephalic co-cultures. *J Neurochem* **73**(6)**:** 2469-2476.

McNaught KS, Perl DP, Brownell AL, Olanow CW (2004). Systemic exposure to proteasome inhibitors causes a progressive model of Parkinson's disease. *Ann Neurol* **56**(1)**:** 149-162.

McNaught KSP, Jenner P (1999b). Altered Glial Function Causes Neuronal Death and Increases Neuronal Susceptibility to 1-Methyl-4-Phenylpyridinium- and 6-Hydroxydopamine-Induced Toxicity in Astrocytic/Ventral Mesencephalic Co-Cultures. *Journal of Neurochemistry* **73**(6)**:** 2469-2476.

McNaught KSP, Jenner P (2001). Proteasomal function is impaired in substantia nigra in Parkinson's disease. *Neuroscience Letters* **297**(3)**:** 191-194.

Meller R, Stevens SL, Minami M, Cameron JA, King S, Rosenzweig H, *et al.* (2005). Neuroprotection by osteopontin in stroke. *J Cereb Blood Flow Metab* **25**(2)**:** 217-225.

Miller DW, Hague SM, Clarimon J, Baptista M, Gwinn-Hardy K, Cookson MR, *et al.* (2004). Alpha-synuclein in blood and brain from familial Parkinson disease with SNCA locus triplication. *Neurology* **62**(10)**:** 1835-1838.

Milner R, Campbell IL (2002). The integrin family of cell adhesion molecules has multiple functions within the CNS. *Journal of neuroscience research* **69**(3)**:** 286-291.

Miyashita M, Ohnishi H, Okazawa H, Tomonaga H, Hayashi A, Fujimoto T-T, *et al.* (2004). Promotion of Neurite and Filopodium Formation by CD47: Roles of Integrins, Rac, and Cdc42 Vol. 15, pp 3950-3963.

Mizuno Y, Ohta S, Tanaka M, Takamiya S, Suzuki K, Sato T, *et al.* (1989). Deficiencies in complex I subunits of the respiratory chain in Parkinson's disease. *Biochem Biophys Res Commun* **163**(3)**:** 1450-1455.

Mogi M, Harada M, Kondo T, Mizuno Y, Narabayashi H, Riederer P, *et al.* (1996). bcl-2 protein is increased in the brain from parkinsonian patients. *Neurosci Lett* **215**(2)**:** 137-139.

Mogi M, Harada M, Kondo T, Riederer P, Inagaki H, Minami M, *et al.* (1994). Interleukin-1[beta], interleukin-6, epidermal growth factor and transforming growth factor-[alpha] are elevated in the brain from parkinsonian patients. *Neuroscience Letters* **180**(2)**:** 147-150.

Mogi M, Togari A, Kondo T, Mizuno Y, Komure O, Kuno S, *et al.* (2000). Caspase activities and tumor necrosis factor receptor R1 (p55) level are elevated in the substantia nigra from parkinsonian brain. *J Neural Transm* **107**(3)**:** 335-341.

Montgomery DL (1994). Astrocytes: form, functions, and roles in disease. *Vet Pathol* **31**(2)**:** 145-167.

Morishita M, Peppas NA (2006). Is the oral route possible for peptide and protein drug delivery? *Drug discovery today* **11**(19-20)**:** 905-910.

Murray J, Taylor SW, Zhang B, Ghosh SS, Capaldi RA (2003). Oxidative damage to mitochondrial complex I due to peroxynitrite: identification of reactive tyrosines by mass spectrometry. *J Biol Chem* **278**(39)**:** 37223-37230.

Murry CE, Giachelli CM, Schwartz SM, Vracko R (1994a). Macrophages express osteopontin during repair of myocardial necrosis Vol. 145, pp 1450-1462.

Murry CE, Giachelli CM, Schwartz SM, Vracko R (1994b). Macrophages express osteopontin during repair of myocardial necrosis. *Am J Pathol* **145**(6)**:** 1450-1462.

Myerowitz R, Lawson D, Mizukami H, Mi Y, Tifft CJ, Proia RL (2002). Molecular pathophysiology in Tay-Sachs and Sandhoff diseases as revealed by gene expression profiling. *Hum Mol Genet* **11**(11)**:** 1343-1350.

Nagakubo D, Taira T, Kitaura H, Ikeda M, Tamai K, Iguchi-Ariga SMM, *et al.* (1997). DJ-1, a Novel Oncogene Which Transforms Mouse NIH3T3 Cells in Cooperation withras. *Biochemical and Biophysical Research Communications* **231**(2)**:** 509-513.

Nagatsu T (2002). Parkinson's disease: changes in apoptosis-related factors suggesting possible gene therapy. *Journal of Neural Transmission* **109**(5)**:** 731-745.

Nagatsu T, Mogi M, Ichinose H, Togari A (2000). Cytokines in Parkinson's disease. *J Neural Transm Suppl*(58)**:** 143-151.

Nakamura Y (2002). Regulating factors for microglial activation. *Biol Pharm Bull* **25**(8)**:** 945-953.

Naor D, Sionov RV, Ish-Shalom D (1997). CD44: structure, function, and association with the malignant process. *Adv Cancer Res* **71:** 241-319.

Narendra D, Tanaka A, Suen DF, Youle RJ (2008). Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *J Cell Biol* **183**(5)**:** 795-803.

Nasu K, Ishida T, Setoguchi M, Higuchi Y, Akizuki S, Yamamoto S (1995). Expression of wild-type and mutated rabbit osteopontin in Escherichia coli, and their effects on adhesion and migration of P388D1 cells. *The Biochemical journal* **307 (Pt 1):** 257-265.

Nau GJ, Chupp GL, Emile JF, Jouanguy E, Berman JS, Casanova JL, *et al.* (2000). Osteopontin expression correlates with clinical outcome in patients with mycobacterial infection. *Am J Pathol* **157**(1)**:** 37-42.

Nau GJ, Guilfoile P, Chupp GL, Berman JS, Kim SJ, Kornfeld H, *et al.* (1997). A chemoattractant cytokine associated with granulomas in tuberculosis and silicosis Vol. 94, pp 6414-6419.

Nau GJ, Liaw L, Chupp GL, Berman JS, Hogan BL, Young RA (1999). Attenuated host resistance against Mycobacterium bovis BCG infection in mice lacking osteopontin. *Infect Immun* **67**(8)**:** 4223-4230.

Nemir M, DeVouge MW, Mukherjee BB (1989). Normal rat kidney cells secrete both phosphorylated and nonphosphorylated forms of osteopontin showing different physiological properties. *J Biol Chem* **264**(30)**:** 18202-18208.

Noiri E, Dickman K, Miller F, Romanov G, Romanov VI, Shaw R, *et al.* (1999). Reduced tolerance to acute renal ischemia in mice with a targeted disruption of the osteopontin gene. *Kidney Int* **56**(1)**:** 74-82.

O'Regan A, Berman JS (2000). Osteopontin: a key cytokine in cell-mediated and granulomatous inflammation. *International journal of experimental pathology* **81**(6)**:** 373-390.

O'Regan AW, Chupp GL, Lowry JA, Goetschkes M, Mulligan N, Berman JS (1999). Osteopontin Is Associated with T Cells in Sarcoid Granulomas and Has T Cell Adhesive and Cytokine-Like Properties In Vitro Vol. 162, pp 1024-1031.

Ochu EE, Rothwell NJ, Waters CM (1998). Caspases mediate 6-hydroxydopamine-induced apoptosis but not necrosis in PC12 cells. *J Neurochem* **70**(6)**:** 2637-2640.

Offen D, Ziv I, Panet H, Wasserman L, Stein R, Melamed E, *et al.* (1997). Dopamine-induced apoptosis is inhibited in PC12 cells expressing Bcl-2. *Cell Mol Neurobiol* **17**(3)**:** 289-304.

Olanow CW (2007). The pathogenesis of cell death in Parkinson's disease – 2007. *Movement Disorders* **22**(S17)**:** S335-S342.

Olanow CW, and McNaught KSP (2006). Ubiquitin-proteasome system and Parkinson's disease. *Movement Disorders* **21**(11)**:** 1806-1823.

Olanow CW, Hauser RA, Gauger L, Malapira T, Koller W, Hubble J, *et al.* (1995). The effect of deprenyl and levodopa on the progression of Parkinson's disease. *Ann Neurol* **38**(5)**:** 771-777.

Olanow CW, Perl DP, DeMartino GN, McNaught KSP (2004). Lewy-body formation is an aggresome-related process: a hypothesis. *The Lancet Neurology* **3**(8)**:** 496-503.

Olanow CW, Rascol O, Hauser R, Feigin PD, Jankovic J, Lang A, *et al.* (2009). A double-blind, delayed-start trial of rasagiline in Parkinson's disease. *N Engl J Med* **361**(13)**:** 1268-1278.

Oldberg A, Franzen A, Heinegard D (1986). Cloning and Sequence Analysis of Rat Bone Sialoprotein (Osteopontin) cDNA Reveals an Arg-Gly-Asp Cell-Binding Sequence. *PNAS* **83**(23)**:** 8819-8823.

Onodera K, Takahashi I, Sasano Y, Bae JW, Mitani H, Kagayama M (2005). Stepwise mechanical stretching inhibits chondrogenesis through cell-matrix adhesion mediated by integrins in embryonic rat limb-bud mesenchymal cells. *European journal of cell biology* **84**(1)**:** 45-58.

Ophascharoensuk V, Giachelli CM, Gordon K, Hughes J, Pichler R, Brown P, *et al.* (1999). Obstructive uropathy in the mouse: Role of osteopontin in interstitial fibrosis and apoptosis. *Kidney Int* **56**(2)**:** 571-580.

Otto D, Unsicker K (1993). FGF-2-mediated protection of cultured mesencephalic dopaminergic neurons against MPTP and MPP+: specificity and impact of culture conditions, non-dopaminergic neurons, and astroglial cells. *Journal of neuroscience research* **34**(4)**:** 382-393.

Outeiro TF, Grammatopoulos TN, Altmann S, Amore A, Standaert DG, Hyman BT, *et al.* (2007). Pharmacological inhibition of PARP-1 reduces alpha-synuclein- and MPP+-induced cytotoxicity in Parkinson's disease in vitro models. *Biochem Biophys Res Commun* **357**(3)**:** 596-602.

Padanilam BJ, Martin DR, Hammerman MR (1996). Insulin-like growth factor I-enhanced renal expression of osteopontin after acute ischemic injury in rats. *Endocrinology* **137**(5)**:** 2133-2140.

Pahwa R, Factor SA, Lyons KE, Ondo WG, Gronseth G, Bronte-Stewart H, *et al.* (2006). Practice Parameter: treatment of Parkinson disease with motor fluctuations and dyskinesia (an evidence-based review): report of the Quality Standards Subcommittee of the American Academy of Neurology. *Neurology* **66**(7)**:** 983-995.

Paisán-RuIz C, Jain S, Evans EW, Gilks WP, Simón J, van der Brug M, *et al.* (2004). Cloning of the Gene Containing Mutations that Cause PARK8-Linked Parkinson's Disease. *Neuron* **44**(4)**:** 595-600.

Palacino JJ, Sagi D, Goldberg MS, Krauss S, Motz C, Wacker M, *et al.* (2004). Mitochondrial dysfunction and oxidative damage in parkin-deficient mice. *J Biol Chem* **279**(18)**:** 18614-18622.

Park TH, Mytilineou C (1992). Protection from 1-methyl-4-phenylpyridinium (MPP+) toxicity and stimulation of regrowth of MPP(+)-damaged dopaminergic fibers by treatment of mesencephalic cultures with EGF and basic FGF. *Brain Res* **599**(1)**:** 83-97.

Parker Jr WD, Parks JK, Swerdlow RH (2008). Complex I deficiency in Parkinson's disease frontal cortex. *Brain Research* **1189:** 215-218.

Parkkinen L, Kauppinen T, Pirttilä T, Autere JM, Alafuzoff I (2005). α-Synuclein pathology does not predict extrapyramidal symptoms or dementia. *Annals of Neurology* **57**(1)**:** 82-91.

Pasinetti GM, Hassler M, Stone D, Finch CE (1999). Glial gene expression during aging in rat striatum and in long-term responses to 6-OHDA lesions. *Synapse* **31**(4)**:** 278-284.

Patarca R, Freeman GJ, Singh RP, Wei FY, Durfee T, Blattner F, *et al.* (1989a). Structural and functional studies of the early T lymphocyte activation 1 (Eta-1) gene. Definition of a novel T cell-dependent response associated with genetic resistance to bacterial infection. *J Exp Med* **170**(1)**:** 145-161.

Patarca R, Freeman GJ, Singh RP, Wei FY, Durfee T, Blattner F, *et al.* (1989b). Structural and functional studies of the early T lymphocyte activation 1 (Eta-1) gene. Definition of a novel T cell-dependent response associated with genetic resistance to bacterial infection Vol. 170, pp 145-161.

Patarca R, Saavedra RA, Cantor H (1993). Molecular and cellular basis of genetic resistance to bacterial infection: the role of the early T-lymphocyte activation-1/osteopontin gene. *Crit Rev Immunol* **13**(3-4)**:** 225-246.

Pauletti GM, Gangwar S, Siahaan TJ, Jeffrey A, T. Borchardt R (1997). Improvement of oral peptide bioavailability: Peptidomimetics and prodrug strategies. *Advanced drug delivery reviews* **27**(2-3)**:** 235-256.

Pearce RKB, Owen A, Daniel S, Jenner P, Marsden CD (1997). Alterations in the distribution of glutathione in the substantia nigra in Parkinson's disease. *Journal of Neural Transmission* **104**(6)**:** 661-677.

Peck D, Isacke CM (1996). CD44 phosphorylation regulates melanoma cell and fibroblast migration on, but not attachment to, a hyaluronan substratum. *Curr Biol* **6**(7)**:** 884-890.

Peluffo H, Gonzalez P, Aris A, Acarin L, Saura J, Villaverde A, *et al.* (2007). RGD domains neuroprotect the immature brain by a glial-dependent mechanism. *Ann Neurol* **62**(3)**:** 251-261.

Peng GS, Li G, Tzeng NS, Chen PS, Chuang DM, Hsu YD, *et al.* (2005). Valproate pretreatment protects dopaminergic neurons from LPS-induced neurotoxicity in rat primary midbrain cultures: role of microglia. *Brain Res Mol Brain Res* **134**(1)**:** 162-169.

Peng J, Mao XO, Stevenson FF, Hsu M, Andersen JK (2004). The herbicide paraquat induces dopaminergic nigral apoptosis through sustained activation of the JNK pathway. *J Biol Chem* **279**(31)**:** 32626-32632.

Pennathur S, Jackson-Lewis V, Przedborski S, Heinecke JW (1999). Mass spectrometric quantification of 3-nitrotyrosine, ortho-tyrosine, and o,o'-dityrosine in brain tissue of 1-methyl-4-phenyl-1,2,3, 6-tetrahydropyridine-treated mice, a model of oxidative stress in Parkinson's disease. *J Biol Chem* **274**(49)**:** 34621-34628.

Perez RG, Hastings TG (2004). Could a loss of α-synuclein function put dopaminergic neurons at risk? *Journal of Neurochemistry* **89**(6)**:** 1318-1324.

Perier C, Tieu K, Guegan C, Caspersen C, Jackson-Lewis V, Carelli V, *et al.* (2005). Complex I deficiency primes Bax-dependent neuronal apoptosis through mitochondrial oxidative damage. *Proc Natl Acad Sci U S A* **102**(52)**:** 19126-19131.

Perry T, Lahiri DK, Chen D, Zhou J, Shaw KT, Egan JM, *et al.* (2002). A novel neurotrophic property of glucagon-like peptide 1: a promoter of nerve growth factor-mediated differentiation in PC12 cells. *J Pharmacol Exp Ther* **300**(3)**:** 958-966.

Persy VP, Verhulst A, Ysebaert DK, De Greef KE, De Broe ME (2003). Reduced postischemic macrophage infiltration and interstitial fibrosis in osteopontin knockout mice. *Kidney Int* **63**(2)**:** 543-553.

Petrovitch H, Ross GW, Abbott RD, Sanderson WT, Sharp DS, Tanner CM, *et al.* (2002). Plantation work and risk of Parkinson disease in a population-based longitudinal study. *Arch Neurol* **59**(11)**:** 1787-1792.

Petrow PK, Hummel KM, Schedel J, Franz JK, Klein CL, Muller-Ladner U, *et al.* (2000). Expression of osteopontin messenger RNA and protein in rheumatoid arthritis: effects of osteopontin on the release of collagenase 1 from articular chondrocytes and synovial fibroblasts. *Arthritis Rheum* **43**(7)**:** 1597-1605.

Pichler R, Giachelli CM, Lombardi D, Pippin J, Gordon K, Alpers CE, *et al.* (1994). Tubulointerstitial disease in glomerulonephritis. Potential role of osteopontin (uropontin). *Am J Pathol* **144**(5)**:** 915-926.

Pickart CM (1997). Targeting of substrates to the 26S proteasome. *FASEB J* **11**(13)**:** 1055-1066.

Pickart CM (2000). Ubiquitin in chains. *Trends Biochem Sci* **25**(11)**:** 544-548.

Pinkstaff JK, Detterich J, Lynch G, Gall C (1999). Integrin Subunit Gene Expression Is Regionally Differentiated in Adult Brain Vol. 19, pp 1541-1556.

Plantman S, Novikova L, Novikov L, Hammarberg H, Wallquist W, Kellerth JO, *et al.* (2005). Integrin messenger RNAs in the red nucleus after axotomy and neurotrophic administration. *Neuroreport* **16**(7)**:** 709-713.

Pollack SB, Linnemeyer PA, Gill S (1994). Induction of osteopontin mRNA expression during activation of murine NK cells Vol. 55, pp 398-400.

Pollard JW (2004). Tumour-educated macrophages promote tumour progression and metastasis. *Nat Rev Cancer* **4**(1)**:** 71-78.

Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, *et al.* (1997). Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science* **276**(5321)**:** 2045-2047.

Possel H, Noack H, Putzke J, Wolf G, Sies H (2000). Selective upregulation of inducible nitric oxide synthase (iNOS) by lipopolysaccharide (LPS) and cytokines in microglia: in vitro and in vivo studies. *Glia* **32**(1)**:** 51-59.

Presgraves SP, Ahmed T, Borwege S, Joyce JN (2004). Terminally differentiated SH-SY5Y cells provide a model system for studying neuroprotective effects of dopamine agonists. *Neurotoxicity research* **5**(8)**:** 579-598.

Previtali SC, Feltri ML, Archelos JJ, Quattrini A, Wrabetz L, Hartung H-P (2001). Role of integrins in the peripheral nervous system. *Progress in Neurobiology* **64**(1)**:** 35-49.

Prince CW (1989). Secondary structure predictions for rat osteopontin. *Connect Tissue Res* **21**(1-4)**:** 15-20.

Priyadarshi A, Khuder SA, Schaub EA, Priyadarshi SS (2001). Environmental risk factors and Parkinson's disease: a metaanalysis. *Environ Res* **86**(2)**:** 122-127.

Prud'Homme GJ, Park CL, Fieser TM, Kofler R, Dixon FJ, Theofilopoulos AN (1983). Identification of a B cell differentiation factor(s) spontaneously produced by proliferating T cells in murine lupus strains of the lpr/lpr genotype Vol. 157, pp 730-742.

PSG (2002a). A controlled trial of rasagiline in early Parkinson disease: the TEMPO Study. *Arch Neurol* **59**(12)**:** 1937-1943.

PSG (2002b). Dopamine transporter brain imaging to assess the effects of pramipexole vs levodopa on Parkinson disease progression. *JAMA* **287**(13)**:** 1653-1661.

PSG (1989). Effect of deprenyl on the progression of disability in early Parkinson's disease. The Parkinson Study Group. *N Engl J Med* **321**(20)**:** 1364-1371.

PSG (1996). Impact of deprenyl and tocopherol treatment on Parkinson's disease in DATATOP patients requiring levodopa. Parkinson Study Group. *Ann Neurol* **39**(1)**:** 37-45.

PSG (2007). Mixed lineage kinase inhibitor CEP-1347 fails to delay disability in early Parkinson disease. *Neurology* **69**(15)**:** 1480-1490.

Qian L, Block ML, Wei S-J, Lin C-f, Reece J, Pang H, *et al.* (2006). Interleukin-10 Protects Lipopolysaccharide-Induced Neurotoxicity in Primary Midbrain Cultures by Inhibiting the Function of NADPH Oxidase. *Journal of Pharmacology and Experimental Therapeutics* **319**(1)**:** 44-52.

Qin L, Wu X, Block ML, Liu Y, Breese GR, Hong J-S, *et al.* (2007). Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. *Glia* **55**(5)**:** 453-462.

Quesada A, Lee BY, Micevych PE (2008). PI3 kinase/Akt activation mediates estrogen and IGF-1 nigral DA neuronal neuroprotection against a unilateral rat model of Parkinson's disease. *Developmental Neurobiology* **68**(5)**:** 632-644.

Reinheckel T, Ullrich O, Sitte N, Grune T (2000). Differential impairment of 20S and 26S proteasome activities in human hematopoietic K562 cells during oxidative stress. *Arch Biochem Biophys* **377**(1)**:** 65-68.

Rezak M (2007). Current pharmacotherapeutic treatment options in Parkinson's disease. *Dis Mon* **53**(4)**:** 214-222.

Rice J, Courter DL, Giachelli CM, Scatena M (2006). Molecular mediators of alphavbeta3-induced endothelial cell survival. *Journal of vascular research* **43**(5)**:** 422-436.

Rittling SR (2011). Osteopontin in macrophage function. *Expert Reviews in Molecular Medicine* **13:** null-null.

Rockwell P, Yuan H, Magnusson R, Figueiredo-Pereira ME (2000). Proteasome Inhibition in Neuronal Cells Induces a Proinflammatory Response Manifested by Upregulation of Cyclooxygenase-2, Its Accumulation as Ubiquitin Conjugates, and Production of the Prostaglandin PGE2. *Archives of Biochemistry and Biophysics* **374**(2)**:** 325-333.

Rodnitzky RL (1999). Can calcium antagonists provide a neuroprotective effect in Parkinson's disease? *Drugs* **57**(6)**:** 845-849.

Rodriguez-Pallares J, Rey P, Parga JA, Muñoz A, Guerra MJ, Labandeira-Garcia JL (2008). Brain angiotensin enhances dopaminergic cell death via microglial activation and NADPH-derived ROS. *Neurobiology of Disease* **31**(1)**:** 58-73.

Rodriguez M, Barroso-Chinea P, Abdala P, Obeso J, Gonzalez-Hernandez T (2001). Dopamine cell degeneration induced by intraventricular administration of 6-

hydroxydopamine in the rat: similarities with cell loss in parkinson's disease. *Exp Neurol* **169**(1)**:** 163-181.

Rollo EE, Denhardt DT (1996a). Differential effects of osteopontin on the cytotoxic activity of macrophages from young and old mice. *Immunology* **88**(4)**:** 642-647.

Rollo EE, Laskin DL, Denhardt DT (1996b). Osteopontin inhibits nitric oxide production and cytotoxicity by activated RAW264.7 macrophages Vol. 60, pp 397-404.

Rosenblad C, Kirik D, Devaux B, Moffat B, Phillips HS, Björklund A (1999). Protection and regeneration of nigral dopaminergic neurons by neurturin or GDNF in a partial lesion model of Parkinson's disease after administration into the striatum or the lateral ventricle. *European Journal of Neuroscience* **11**(5)**:** 1554-1566.

Rott R, Szargel R, Haskin J, Shani V, Shainskaya A, Manov I, *et al.* (2008). Monoubiquitylation of alpha-synuclein by seven in absentia homolog (SIAH) promotes its aggregation in dopaminergic cells. *J Biol Chem* **283**(6)**:** 3316-3328.

Rubinsztein DC (2006). The roles of intracellular protein-degradation pathways in neurodegeneration. *Nature* **443**(7113)**:** 780-786.

Saigoh K, Wang YL, Suh JG, Yamanishi T, Sakai Y, Kiyosawa H, *et al.* (1999). Intragenic deletion in the gene encoding ubiquitin carboxy-terminal hydrolase in gad mice. *Nat Genet* **23**(1)**:** 47-51.

Salih E, Wang J, Mah J, Fluckiger R (2002). Natural variation in the extent of phosphorylation of bone phosphoproteins as a function of in vivo new bone formation induced by demineralized bone matrix in soft tissue and bony environments. *The Biochemical journal* **364**(Pt 2)**:** 465-474.

Sanchez-Pernaute R, Ferree A, Cooper O, Yu M, Brownell AL, Isacson O (2004). Selective COX-2 inhibition prevents progressive dopamine neuron degeneration in a rat model of Parkinson's disease. *Journal of neuroinflammation* **1**(1)**:** 6.

Saner A, Thoenen H (1971). Model Experiments on the Molecular Mechanism of Action of 6-Hydroxydopamine. *Molecular Pharmacology* **7**(2)**:** 147-154.

Saporito MS, Thomas BA, Scott RW (2000). MPTP activates c-Jun NH(2)-terminal kinase (JNK) and its upstream regulatory kinase MKK4 in nigrostriatal neurons in vivo. *J Neurochem* **75**(3)**:** 1200-1208.

Satoh J, Obayashi S, Misawa T, Tabunoki H, Yamamura T, Arima K, *et al.* (2008). Neuromyelitis optica/Devic's disease: gene expression profiling of brain lesions. *Neuropathology* **28**(6)**:** 561-576.

Scatena M, Almeida M, Chaisson ML, Fausto N, Nicosia RF, Giachelli CM (1998). NF-kappa B Mediates alpha vbeta 3 Integrin-induced Endothelial Cell Survival Vol. 141, pp 1083-1093.

Scatena M, Liaw L, Giachelli CM (2007). Osteopontin: a multifunctional molecule regulating chronic inflammation and vascular disease. *Arterioscler Thromb Vasc Biol* **27**(11)**:** 2302-2309.

Schapira AH (2002). Dopamine agonists and neuroprotection in Parkinson's disease. *Eur J Neurol* **9 Suppl 3:** 7-14.

Schapira AH (2008a). Mitochondria in the aetiology and pathogenesis of Parkinson's disease. *Lancet Neurol* **7**(1)**:** 97-109.

Schapira AH (2009a). Molecular and clinical pathways to neuroprotection of dopaminergic drugs in Parkinson disease. *Neurology* **72**(7 Suppl)**:** S44-50.

Schapira AH (2009b). Neurobiology and treatment of Parkinson's disease. *Trends in pharmacological sciences* **30**(1)**:** 41-47.

Schapira AH (2008b). Progress in neuroprotection in Parkinson's disease. *Eur J Neurol* **15 Suppl 1:** 5-13.

Schapira AH, Cooper JM, Dexter D, Clark JB, Jenner P, Marsden CD (1990). Mitochondrial complex I deficiency in Parkinson's disease. *J Neurochem* **54**(3)**:** 823-827.

Schapira AHV, Albrecht S, Barone P, Comella CL, McDermott MP, Mizuno Y, *et al.* (2010). Rationale for delayed-start study of pramipexole in Parkinson's disease: The PROUD study. *Movement Disorders* **25**(11)**:** 1627-1632.

Schroeter M, Zickler P, Denhardt DT, Hartung HP, Jander S (2006). Increased thalamic neurodegeneration following ischaemic cortical stroke in osteopontin-deficient mice. *Brain* **129**(Pt 6)**:** 1426-1437.

Scott JA, Weir ML, Wilson SM, Xuan JW, Chambers AF, McCormack DG (1998). Osteopontin inhibits inducible nitric oxide synthase activity in rat vascular tissue Vol. 275, pp H2258-2265.

Seidler A, Hellenbrand W, Robra BP, Vieregge P, Nischan P, Joerg J, *et al.* (1996). Possible environmental, occupational, and other etiologic factors for Parkinson's disease: a case-control study in Germany. *Neurology* **46**(5)**:** 1275-1284.

Semchuk KM, Love EJ, Lee RG (1992). Parkinson's disease and exposure to agricultural work and pesticide chemicals. *Neurology* **42**(7)**:** 1328-1335.

Senger DR, Perruzzi CA, Papadopoulos-Sergiou A, Van de Water L (1994). Adhesive properties of osteopontin: regulation by a naturally occurring thrombin-cleavage in close proximity to the GRGDS cell-binding domain. *Mol. Biol. Cell* **5**(5)**:** 565-574.

Senger DR, Perruzzi CA, Papadopoulos A, Tenen DG (1989). Purification of a human milk protein closely similar to tumor-secreted phosphoproteins and

osteopontin. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology* **996**(1-2)**:** 43-48.

Senger DR, Wirth DF, Hynes RO (1979). Transformed mammalian cells secrete specific proteins and phosphoproteins. *Cell* **16**(4)**:** 885-893.

Shamoto-Nagai M, Maruyama W, Kato Y, Isobe K, Tanaka M, Naoi M, *et al.* (2003). An inhibitor of mitochondrial complex I, rotenone, inactivates proteasome by oxidative modification and induces aggregation of oxidized proteins in SH-SY5Y cells. *Journal of neuroscience research* **74**(4)**:** 589-597.

Sharif SA, Du X, Myles T, Song JJ, Price E, Lee DM, *et al.* (2009). Thrombin-activatable carboxypeptidase B cleavage of osteopontin regulates neutrophil survival and synoviocyte binding in rheumatoid arthritis. *Arthritis & Rheumatism* **60**(10)**:** 2902-2912.

Sheehan JP, Palmer PE, Helm GA, Tuttle JB (1997). MPP+ induced apoptotic cell death in SH-SY5Y neuroblastoma cells: an electron microscope study. *Journal of neuroscience research* **48**(3)**:** 226-237.

Sherer TB, Betarbet R, Kim JH, Greenamyre JT (2003a). Selective microglial activation in the rat rotenone model of Parkinson's disease. *Neurosci Lett* **341**(2)**:** 87-90.

Sherer TB, Kim JH, Betarbet R, Greenamyre JT (2003b). Subcutaneous rotenone exposure causes highly selective dopaminergic degeneration and alpha-synuclein aggregation. *Exp Neurol* **179**(1)**:** 9-16.

Shimoda K, Sauve Y, Marini A, Schwartz JP, Commissiong JW (1992). A high percentage yield of tyrosine hydroxylase-positive cells from rat E14 mesencephalic cell culture. *Brain Res* **586**(2)**:** 319-331.

Shin T, Ahn M, Kim H, Moon C, Kang TY, Lee JM, *et al.* (2005). Temporal expression of osteopontin and CD44 in rat brains with experimental cryolesions. *Brain Res* **1041**(1)**:** 95-101.

Shiraga H, Min W, VanDusen WJ, Clayman MD, Miner D, Terrell CH, *et al.* (1992). Inhibition of Calcium Oxalate Crystal Growth in vitro by Uropontin: Another Member of the Aspartic Acid-Rich Protein Superfamily. *PNAS* **89**(1)**:** 426-430.

Shults CW, Oakes D, Kieburtz K, Beal MF, Haas R, Plumb S, *et al.* (2002). Effects of coenzyme Q10 in early Parkinson disease: evidence of slowing of the functional decline. *Arch Neurol* **59**(10)**:** 1541-1550.

Silva RM, Kuan C-Y, Rakic P, Burke RE (2005). Mixed lineage kinase–c-jun N-terminal kinase signaling pathway: A new therapeutic target in Parkinson's disease. *Movement Disorders* **20**(6)**:** 653-664.

Singer CA, Rogers KL, Dorsa DM (1998). Modulation of Bcl-2 expression: a potential component of estrogen protection in NT2 neurons. *Neuroreport* **9**(11)**:** 2565-2568.

Singer TP, Castagnoli N, Jr., Ramsay RR, Trevor AJ (1987). Biochemical events in the development of parkinsonism induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *J Neurochem* **49**(1)**:** 1-8.

Singh K, DeVouge MW, Mukherjee BB (1990). Physiological properties and differential glycosylation of phosphorylated and nonphosphorylated forms of osteopontin secreted by normal rat kidney cells. *Journal of Biological Chemistry* **265**(30)**:** 18696-18701.

Singh N, Pillay V, Choonara YE (2007). Advances in the treatment of Parkinson's disease. *Prog Neurobiol* **81**(1)**:** 29-44.

Smith JH, Denhardt DT (1987). Molecular cloning of a tumor promoter-inducible mRNA found in JB6 mouse epidermal cells: induction is stable at high, but not at low, cell densities. *Journal of cellular biochemistry* **34**(1)**:** 13-22.

Smith LL, Cheung H-K, Ling LE, Chen J, Sheppard D, Pytela R, *et al.* (1996). Osteopontin N-terminal Domain Contains a Cryptic Adhesive Sequence Recognized by alpha 9beta 1 Integrin Vol. 271, pp 28485-28491.

Smith LL, Giachelli CM (1998). Structural Requirements for [alpha]9[beta]1-Mediated Adhesion and Migration to Thrombin-Cleaved Osteopontin. *Experimental Cell Research* **242**(1)**:** 351-360.

Sodek J, Ganss B, McKee MD (2000). Osteopontin. *Crit Rev Oral Biol Med* **11**(3)**:** 279-303.

Sofic E, Lange KW, Jellinger K, Riederer P (1992). Reduced and oxidized glutathione in the substantia nigra of patients with Parkinson's disease. *Neuroscience Letters* **142**(2)**:** 128-130.

Sørensen ES, Petersen TE, Højrup P (1995). Posttranslational modifications of bovine osteopontin: Identification of twenty-eight phosphorylation and three O-glycosylation sites. *Protein Science* **4**(10)**:** 2040-2049.

Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M (1997). Alpha-synuclein in Lewy bodies. *Nature* **388**(6645)**:** 839-840.

Sriram K, Pai KS, Boyd MR, Ravindranath V (1997). Evidence for generation of oxidative stress in brain by MPTP: in vitro and in vivo studies in mice. *Brain Res* **749**(1)**:** 44-52.

St. P. McNaught K, Belizaire R, Isacson O, Jenner P, Olanow CW (2003). Altered Proteasomal Function in Sporadic Parkinson's Disease. *Experimental Neurology* **179**(1)**:** 38-46.

Stallcup WB (1979). Sodium and calcium fluxes in a clonal nerve cell line Vol. 286, pp 525-540.

Stefanis L, Larsen KE, Rideout HJ, Sulzer D, Greene LA (2001). Expression of A53T mutant but not wild-type alpha-synuclein in PC12 cells induces alterations of the ubiquitin-dependent degradation system, loss of dopamine release, and autophagic cell death. *J Neurosci* **21**(24)**:** 9549-9560.

Storch A, Burkhardt K, Ludolph AC, Schwarz J (2000). Protective Effects of Riluzole on Dopamine Neurons. *Journal of Neurochemistry* **75**(6)**:** 2259-2269.

Streeter PR, Berg EL, Rouse BT, Bargatze RF, Butcher EC (1988). A tissue-specific endothelial cell molecule involved in lymphocyte homing. *Nature* **331**(6151)**:** 41-46.

Sun F, Anantharam V, Zhang D, Latchoumycandane C, Kanthasamy A, Kanthasamy AG (2006). Proteasome inhibitor MG-132 induces dopaminergic degeneration in cell culture and animal models. *Neurotoxicology* **27**(5)**:** 807-815.

Sveinbjornsdottir S, Hicks AA, Jonsson T, Petursson H, Gugmundsson G, Frigge ML, *et al.* (2000). Familial aggregation of Parkinson's disease in Iceland. *N Engl J Med* **343**(24)**:** 1765-1770.

Swerdlow RH, Parks JK, Miller SW, Tuttle JB, Trimmer PA, Sheehan JP, *et al.* (1996). Origin and functional consequences of the complex I defect in Parkinson's disease. *Ann Neurol* **40**(4)**:** 663-671.

Sy MS, Guo YJ, Stamenkovic I (1991). Distinct effects of two CD44 isoforms on tumor growth in vivo. *J Exp Med* **174**(4)**:** 859-866.

Takahashi-Niki K, Niki T, Taira T, Iguchi-Ariga SM, Ariga H (2004). Reduced anti-oxidative stress activities of DJ-1 mutants found in Parkinson's disease patients. *Biochem Biophys Res Commun* **320**(2)**:** 389-397.

Takai N, Nakanishi H, Tanabe K, Nishioku T, Sugiyama T, Fujiwara M, *et al.* (1998). Involvement of caspase-like proteinases in apoptosis of neuronal PC12 cells and primary cultured microglia induced by 6-hydroxydopamine. *Journal of neuroscience research* **54**(2)**:** 214-222.

Tatton NA (2000). Increased caspase 3 and Bax immunoreactivity accompany nuclear GAPDH translocation and neuronal apoptosis in Parkinson's disease. *Exp Neurol* **166**(1)**:** 29-43.

Tatton NA, Kish SJ (1997). In situ detection of apoptotic nuclei in the substantia nigra compacta of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice using terminal deoxynucleotidyl transferase labelling and acridine orange staining. *Neuroscience* **77**(4)**:** 1037-1048.

Taylor DL, Diemel LT, Pocock JM (2003). Activation of microglial group III metabotropic glutamate receptors protects neurons against microglial neurotoxicity. *J Neurosci* **23**(6)**:** 2150-2160.

Thalmann GN, Sikes RA, Devoll RE, Kiefer JA, Markwalder R, Klima I, *et al.* (1999). Osteopontin: Possible Role in Prostate Cancer Progression. *Clin Cancer Res* **5**(8)**:** 2271-2277.

Tomac A, Lindqvist E, Lin LF, Ogren SO, Young D, Hoffer BJ, *et al.* (1995). Protection and repair of the nigrostriatal dopaminergic system by GDNF in vivo. *Nature* **373**(6512)**:** 335-339.

Tomás-Camardiel M, Rite I, Herrera AJ, de Pablos RM, Cano J, Machado A, *et al.* (2004). Minocycline reduces the lipopolysaccharide-induced inflammatory reaction, peroxynitrite-mediated nitration of proteins, disruption of the blood-brain barrier, and damage in the nigral dopaminergic system. *Neurobiology of Disease* **16**(1)**:** 190-201.

Tomaselli KJ, Hall DE, Flier LA, Gehlsen KR, Turner DC, Carbonetto S, *et al.* (1990). A neuronal cell line (PC12) expresses two [beta]1-class integrins--[alpha]1[beta]1, and [alpha]3[beta]1--that recognize different neurite outgrowth-promoting domains in laminin. *Neuron* **5**(5)**:** 651-662.

Tompkins MM, Basgall EJ, Zamrini E, Hill WD (1997). Apoptotic-like changes in Lewy-body-associated disorders and normal aging in substantia nigral neurons. *Am J Pathol* **150**(1)**:** 119-131.

Trochon V, Mabilat C, Bertrand P, Legrand Y, Smadja-Joffe F, Soria C, *et al.* (1996). Evidence of involvement of CD44 in endothelial cell proliferation, migration and angiogenesis in vitro. *Int J Cancer* **66**(5)**:** 664-668.

Trueblood NA, Xie Z, Communal C, Sam F, Ngoy S, Liaw L, *et al.* (2001). Exaggerated Left Ventricular Dilation and Reduced Collagen Deposition After Myocardial Infarction in Mice Lacking Osteopontin Vol. 88, pp 1080-1087.

Tseng JL, Bruhn SL, Zurn AD, Aebischer P (1998). Neurturin protects dopaminergic neurons following medial forebrain bundle axotomy. *Neuroreport* **9**(8)**:** 1817-1822.

Underhill C (1992). CD44: the hyaluronan receptor. *J Cell Sci* **103 (Pt 2):** 293-298.

Valente EM, Abou-Sleiman PM, Caputo V, Muqit MM, Harvey K, Gispert S, *et al.* (2004). Hereditary early-onset Parkinson's disease caused by mutations in PINK1. *Science* **304**(5674)**:** 1158-1160.

Valente EM, Bentivoglio AR, Dixon PH, Ferraris A, Ialongo T, Frontali M, *et al.* (2001). Localization of a novel locus for autosomal recessive early-onset parkinsonism, PARK6, on human chromosome 1p35-p36. *Am J Hum Genet* **68**(4)**:** 895-900.

van Dijk S, D'Errico JA, Somerman MJ, Farach-Carson MC, Butler WT (1993). Evidence that a non-RGD domain in rat osteopontin is involved in cell attachment. *Journal of Bone and Mineral Research* **8**(12)**:** 1499-1506.

Vila M, Jackson-Lewis V, Guegan C, Wu DC, Teismann P, Choi DK, *et al.* (2001a). The role of glial cells in Parkinson's disease. *Curr Opin Neurol* **14**(4)**:** 483-489.

Vila M, Jackson-Lewis V, Vukosavic S, Djaldetti R, Liberatore G, Offen D, *et al.* (2001b). Bax ablation prevents dopaminergic neurodegeneration in the 1-methyl- 4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease. *Proc Natl Acad Sci U S A* **98**(5)**:** 2837-2842.

Viswanath V, Wu Y, Boonplueang R, Chen S, Stevenson FF, Yantiri F, *et al.* (2001). Caspase-9 activation results in downstream caspase-8 activation and bid cleavage in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced Parkinson's disease. *J Neurosci* **21**(24)**:** 9519-9528.

Vogiatzi T, Xilouri M, Vekrellis K, Stefanis L (2008). Wild type alpha-synuclein is degraded by chaperone-mediated autophagy and macroautophagy in neuronal cells. *J Biol Chem* **283**(35)**:** 23542-23556.

von Gertten C, Flores Morales A, Holmin S, Mathiesen T, Nordqvist AC (2005). Genomic responses in rat cerebral cortex after traumatic brain injury. *BMC Neurosci* **6:** 69.

Vyas S, Javoy-Agid F, Herrero MT, Strada O, Boissiere F, Hibner U, *et al.* (1997). Expression of Bcl-2 in adult human brain regions with special reference to neurodegenerative disorders. *J Neurochem* **69**(1)**:** 223-231.

Wang H, Zhan Y, Xu L, Feuerstein GZ, Wang X (2001). Use of Suppression Subtractive Hybridization for Differential Gene Expression in Stroke : Discovery of CD44 Gene Expression and Localization in Permanent Focal Stroke in Rats. *Stroke* **32**(4)**:** 1020-1027.

Wang JY, Grabacka M, Marcinkiewicz C, Staniszewska I, Peruzzi F, Khalili k, *et al.* (2006). Involvement of ?1?1 integrin in insulin-like growth factor-1-mediated protection of PC12 neuronal processes from tumor necrosis factor-?-induced injury Vol. 83, pp 7-18.

Wang X, Louden C, Yue TL, Ellison JA, Barone FC, Solleveld HA, *et al.* (1998). Delayed expression of osteopontin after focal stroke in the rat. *J Neurosci* **18**(6)**:** 2075-2083.

Webb DS, Shimizu Y, Van Seventer GA, Shaw S, Gerrard TL (1990). LFA-3, CD44, and CD45: physiologic triggers of human monocyte TNF and IL-1 release. *Science* **249**(4974)**:** 1295-1297.

Webb JL, Ravikumar B, Atkins J, Skepper JN, Rubinsztein DC (2003). Alpha-Synuclein is degraded by both autophagy and the proteasome. *J Biol Chem* **278**(27)**:** 25009-25013.

Weber GF (2001). The metastasis gene osteopontin: a candidate target for cancer therapy. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer* **1552**(2)**:** 61-85.

Weber GF, Ashkar S, Glimcher MJ, Cantor H (1996a). Receptor-ligand interaction between CD44 and osteopontin (Eta-1). *Science* **271**(5248)**:** 509-512.

Weber GF, Cantor H (1996b). The immunology of Eta-1/osteopontin. *Cytokine Growth Factor Rev* **7**(3)**:** 241-248.

Wesson JA, Johnson RJ, Mazzali M, Beshensky AM, Stietz S, Giachelli C, *et al.* (2003). Osteopontin Is a Critical Inhibitor of Calcium Oxalate Crystal Formation and Retention in Renal Tubules. *J Am Soc Nephrol* **14**(1)**:** 139-147.

West AB, Moore DJ, Biskup S, Bugayenko A, Smith WW, Ross CA, *et al.* (2005). Parkinson's disease-associated mutations in leucine-rich repeat kinase 2 augment kinase activity. *Proc Natl Acad Sci U S A* **102**(46)**:** 16842-16847.

Whone AL, Watts RL, Stoessl AJ, Davis M, Reske S, Nahmias C, *et al.* (2003). Slower progression of Parkinson's disease with ropinirole versus levodopa: The REAL-PET study. *Ann Neurol* **54**(1)**:** 93-101.

Wirths O, Breyhan H, Marcello A, Cotel MC, Bruck W, Bayer TA (2010). Inflammatory changes are tightly associated with neurodegeneration in the brain and spinal cord of the APP/PS1KI mouse model of Alzheimer's disease. *Neurobiol Aging* **31**(5)**:** 747-757.

Wojtera M, Sikorska B, Sobow T, Liberski PP (2005). Microglial cells in neurodegenerative disorders. *Folia Neuropathol* **43**(4)**:** 311-321.

Wrana JL, Kubota T, Zhang Q, Overall CM, Aubin JE, Butler WT, *et al.* (1991). Regulation of transformation-sensitive secreted phosphoprotein (SPPI/osteopontin) expression by transforming growth factor-beta. Comparisons with expression of SPARC (secreted acidic cysteine-rich protein). *The Biochemical journal* **273 (Pt 3):** 523-531.

Wu DC, Jackson-Lewis V, Vila M, Tieu K, Teismann P, Vadseth C, *et al.* (2002). Blockade of microglial activation is neuroprotective in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson disease. *J Neurosci* **22**(5)**:** 1763-1771.

Wung JK, Perry G, Kowalski A, Harris PL, Bishop GM, Trivedi MA, *et al.* (2007). Increased expression of the remodeling- and tumorigenic-associated factor osteopontin in pyramidal neurons of the Alzheimer's disease brain. *Curr Alzheimer Res* **4**(1)**:** 67-72.

Xilouri M, Vogiatzi T, Vekrellis K, Park D, Stefanis L (2009). Abberant alpha-synuclein confers toxicity to neurons in part through inhibition of chaperone-mediated autophagy. *PLoS One* **4**(5)**:** e5515.

Yan P, Mühlethaler A, Balmas k, Maja B, Beck N, Gross N (2003). Hypermethylation-mediated regulation of <I>CD44</I> gene expression in human neuroblastoma Vol. 36, pp 129-138.

Yang F, Yang YP, Mao CJ, Cao BY, Cai ZL, Shi JJ, *et al.* (2009). Role of autophagy and proteasome degradation pathways in apoptosis of PC12 cells overexpressing human alpha-synuclein. *Neurosci Lett* **454**(3)**:** 203-208.

Yang L, Matthews RT, Schulz JB, Klockgether T, Liao AW, Martinou JC, *et al.* (1998). 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyride neurotoxicity is attenuated in mice overexpressing Bcl-2. *J Neurosci* **18**(20)**:** 8145-8152.

Yang Q, Mao Z (2010). Parkinson Disease: A Role for Autophagy? *The Neuroscientist* **16**(4)**:** 335-341.

Yang Y, Wang J, Xu C, Pan H, Zhang Z (2006). Maltol inhibits apoptosis of human neuroblastoma cells induced by hydrogen peroxide. *Journal of biochemistry and molecular biology* **39**(2)**:** 145-149.

Yokasaki Y, Sheppard D (2000). Mapping of the Cryptic Integrin-Binding Site in Osteopontin Suggests a New Mechanism by Which Thrombin Can Regulate Inflammation and Tissue Repair. *Trends in Cardiovascular Medicine* **10**(4)**:** 155-159.

Yokosaki Y, Tanaka K, Higashikawa F, Yamashita K, Eboshida A (2005). Distinct structural requirements for binding of the integrins alphavbeta6, alphavbeta3, alphavbeta5, alpha5beta1 and alpha9beta1 to osteopontin. *Matrix Biol* **24**(6)**:** 418-427.

Yoritaka A, Hattori N, Uchida K, Tanaka M, Stadtman ER, Mizuno Y (1996). Immunohistochemical detection of 4-hydroxynonenal protein adducts in Parkinson disease. *PNAS* **93**(7)**:** 2696-2701.

Yoshitake H, Rittling SR, Denhardt DT, Noda M (1999). Osteopontin-deficient mice are resistant to ovariectomy-induced bone resorption. *Proc Natl Acad Sci U S A* **96**(14)**:** 8156-8160.

Yu Q, Toole BP (1997). Common pattern of CD44 isoforms is expressed in morphogenetically active epithelia. *Dev Dyn* **208**(1)**:** 1-10.

Yue T-L, McKenna PJ, Ohlstein EH, Farach-Carson MC, Butler WT, Johanson K, *et al.* (1994). Osteopontin-Stimulated Vascular Smooth Muscle Cell Migration Is Mediated by [beta]3 Integrin. *Experimental Cell Research* **214**(2)**:** 459-464.

Zarow C, Lyness SA, Mortimer JA, Chui HC (2003). Neuronal Loss Is Greater in the Locus Coeruleus Than Nucleus Basalis and Substantia Nigra in Alzheimer and Parkinson Diseases. *Arch Neurol* **60**(3)**:** 337-341.

Zhang J, Perry G, Smith MA, Robertson D, Olson SJ, Graham DG, *et al.* (1999). Parkinson's Disease Is Associated with Oxidative Damage to Cytoplasmic DNA and RNA in Substantia Nigra Neurons. *Am J Pathol* **154**(5)**:** 1423-1429.

Zhu JH, Guo F, Shelburne J, Watkins S, Chu CT (2003). Localization of phosphorylated ERK/MAP kinases to mitochondria and autophagosomes in Lewy body diseases. *Brain Pathol* **13**(4)**:** 473-481.

Zhu JH, Horbinski C, Guo F, Watkins S, Uchiyama Y, Chu CT (2007). Regulation of autophagy by extracellular signal-regulated protein kinases during 1-methyl-4-phenylpyridinium-induced cell death. *Am J Pathol* **170**(1)**:** 75-86.

Zimprich A, Biskup S, Leitner P, Lichtner P, Farrer M, Lincoln S, *et al.* (2004). Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology. *Neuron* **44**(4)**:** 601-607.

Zuch CL, Nordstroem VK, Briedrick LA, Hoernig GR, Granholm AC, Bickford PC (2000). Time course of degenerative alterations in nigral dopaminergic neurons following a 6-hydroxydopamine lesion. *J Comp Neurol* **427**(3)**:** 440-454.